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Regulation of DNA Replication Initiation by Histone Acetylation and the DNA Unwinding Element Binding Protein DUE-B

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REGULATION OF DNA REPLICATION INITIATON BY HISTONE ACETYLATION AND THE DNA UNWINDING ELEMENT BINDING PROTEIN DUE-B

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By

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Michael G. Kemp ENTITLED Regulation of DNA Replication Initiation by Histone Acetylation and the DNA Unwinding Element Binding Protein DUE-B BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Kemp, Michael G. Ph.D., Biomedical Sciences Program, Wright State University, 2006. Regulation of DNA Replication Initiation by Histone Acetylation and the DNA Unwinding Element Binding Protein DUE-B.

Duplication of the genome during S phase of the mitotic cell cycle begins at thousands of sites along chromosomes termed origins of replication. Although many of the essential protein components catalyzing events at these sites are known and are conserved throughout eukaryotes, the likelihood or efficiency of initiation of DNA synthesis at any given genomic site is expected to be influenced by other novel factors, including aspects of chromatin and DNA structure.

Here I show that increased histone H4 acetylation at replication origin loci occurs after treatment with the histone deacetylase inhibitor TSA and coincides with a loss of specific initiation site selection both within origin loci and throughout the genome. Furthermore, new replication initiation sites become activated or used with greater frequency after treatment with TSA, and TSA promotes the activation of replication origins earlier during the S phase of the cell cycle. These data suggest a physiological role for histone acetylation in controlling the initiation of DNA synthesis at specific chromosomal sites.

Regions of helically unstable DNA termed DNA unwinding elements (DUEs) are commonly found at replication origins, and our laboratory identified a DUE-binding protein (DUE-B) using the c-myc DUE in a yeast one-hybrid screen. Here I demonstrate that DUE-B is required for efficient entry into S phase in human cells and for efficient

replication in the *Xenopus* egg extract replication system. Structural analyses show the N-terminal portion of the protein to be identical to that of bacterial D-aminoacyl-tRNA deacylases. Human DUE-B possesses this function *in vitro* and the ability to hydrolyze ATP, suggesting that DUE-B may be a multifunctional esterase. Unique to vertebrate homologs of DUE-B is a C-terminal extension of 62 amino acids that binds DNA and is targeted for phosphorylation by CK2. The addition of the C-terminal domain to DUE-B in higher eukaryotes may have coincided during evolution with the development of a novel function for this protein in the initiation of DNA replication.

Together these two sets of data argue that previously uncharacterized factors regulate the initiation of DNA replication in higher eukaryotes, possibly to deal with the complex chromosomal architecture found in these organisms.

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DEDICATION

To my wife Emily,

Whose constant love, support, and understanding made this dissertation possible.

And also to Andrew and Evelyn,

May your lives be filled with the joy and inspiration that come from learning and discovery.

INTRODUCTION

Genome duplication is an intricately regulated process of the cell division cycle, where a loss of the physical integrity of chromosomes or inaccurately copied DNA can result in lethality for a cell or organism. In mammalian cells, such defects may also lead to cancer. Numerous controls have thus evolved to ensure genome stability, many of which play important roles during the DNA synthesis phase of the cell cycle. This phase of the cell cycle begins with the initiation of DNA replication at thousands of sites throughout the genome in human somatic cells. Misregulation of this replication initiation step may give rise to gene amplification or other potentially genome destabilizing effects, and thus a primary mechanism of maintaining genome stability is the proper selection and control of initiation sites along chromosomes from which to begin DNA synthesis.

An understanding of precisely how initiation sites for DNA replication are selected was originally formulated in the replicon hypothesis (Jacob and Brenner, 1963), which proposed that *trans*-acting factors called initiators bind to specific, *cis*-acting DNA sequences termed replicators, and then recruit the additional factors needed for chromosome duplication. Although originally proposed for the small, circular bacterial chromosome, this model has been extended to include the replication of larger, more complex genomes of eukaryotic organisms. The general principle is that the binding of one or more initiator proteins to specific chromosomal sites in the genome defines the sites on which the machinery required for replication will be assembled and start DNA synthesis. Generally, the essential factors of this machinery include a helicase to unwind the DNA and a DNA polymerase to copy the unwound DNA template. Furthermore, proteins involved in loading the helicase at the replication origin, in stabilizing the unwound template DNA, and in loading polymerase co-factors are found in all experimental systems studied. Thus the general strategy and mechanism by which genome duplication begins appears to be largely conserved throughout evolution.

Licensing the replication origin

The first step in the initiation of DNA synthesis is defining the sites along chromosomes from which DNA replication will begin. These sites often share conserved functional elements, especially in budding yeast where all replication origins have now been identified by microarray analyses (Raghuraman et al, 2001; Wyrick et al, 2001). Although replication origins have been relatively easy to identify in

budding yeast, replicator elements in metazoan species have been more difficult to identify and characterize (Aladjem and Fanning, 2004; Gilbert, 2004). A more detailed overview of replicators is discussed later.

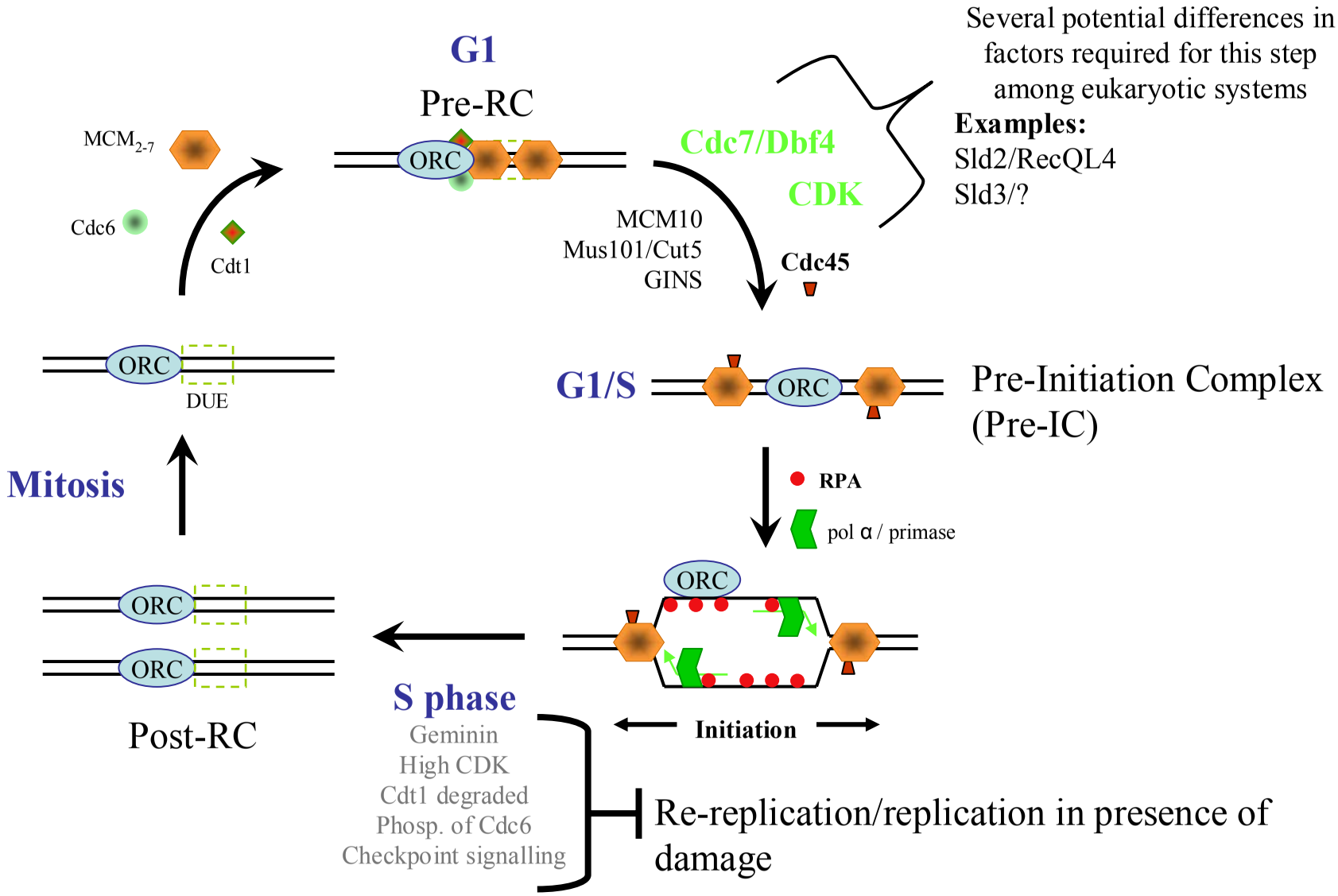
Regardless of how these replication origin sites are selected, a basic set of conserved protein factors are found in all eukaryotes that work to license genomic sites for replication initiation (Figure 1). Importantly, this licensing is finely regulated to allow one and only one initiation at the origin per S phase in normal cells.

Evidence from a variety of experimental systems has pointed towards a group of proteins termed the origin recognition complex (ORC) as a primary eukaryotic replication initiation factor required for the replication of chromosomal DNA. First discovered in budding yeast in the early 1990's (Bell and Stillman, 1992) the ORC is comprised of six interacting subunits (Orc1-6) that define the sites along chromosomes from which replication can initiate (DePamphilis, 2005). Homologs of the ORC subunits have since been identified in all eukaryotic replication model systems, and an evolutionary homolog of one subunit, Orc1, has even been described in archaea. Similar to initiators of bacteria, the Orc1, Orc4, and Orc5 subunits belong to the AAA^+ family of ATPases (Neuwald et al, 1999). In budding yeast, microarray analysis has identified all the sites of ORC binding throughout the genome, and these data show a strong correlation between ORC binding and replication initiation. Although ORC binds chromatin and is required for DNA replication initiation in *Xenopus*, *Drosophila*, and mammalian cells, knowledge of the specific sites of ORC binding in metazoans is much more limited. Regardless of how ORC is recruited to origin sites, ORC is likely regulated by conserved mechanisms such as phosphorylation and ATP hydrolysis to prevent re-formation of replication complexes after origins have already initiated DNA synthesis (Remus et al, 2005).

A second important regulator of origin licensing is the protein Cdc6, a protein that shares an evolutionary ancestor with Orc1, based on sequence similarity and evidence that both are members of the AAA^+ family of ATPases. Indeed in archaea, a single Orc1/Cdc6 ortholog appears to function as the initiator protein (Neuwald et al, 1999). Through genetic and biochemical studies, Cdc6 is required for DNA synthesis and for entry into the S phase of the cell cycle (Bell and Dutta, 2002), and this activity results from its ATP hydrolysis. Cdc6 interacts with the ORC *in vivo,* and in *Xenopus* egg extracts Cdc6 regulates

Figure 1. Replication protein assembly in metazoans.

A model for the stepwise assembly of replication complexes at origins of replication is provided. Pre-replication complexes (pre-RCs) form along chromsomes during G1 phase of the cell cycle, while CDK levels are low. CDK and DDK activity promote the activities of several other proteins that lead to the recruitment of Cdc45 and the GINS complex to the origin, forming a pre-initiation complex (pre-IC). With this recruitment and kinase activity comes activation of the MCM helicase activity and then unwinding of the origin DNA. The single-stranded DNA that is generated is bound by RPA, stabilizing the template for priming and DNA synthesis by DNA polymerase alpha/primase.


G1
Pre-RC
MCM2-7
Cdc6
Cdt1
ORC
Cdc7/Dbf4
CDK
MCM10
Mus101/Cut5
GINS
Cdc45
Several potential differences in factors required for this step among eukaryotic systems
Examples:
Sld2/RecQL4
Sld3/?
G1/S
Pre-Initiation Complex (Pre-IC)
RPA
pol α / primase
Initiation
S phase
Geminin
High CDK
Cdt1 degraded
Phosp. of Cdc6
Checkpoint signalling
Re-replication/replication in presence of damage
Post-RC
DUE
Mitosis

the stability of ORC on chromatin (Harvey and Newport, 2003), indicating origin selection in eukaryotes may be primarily dependent upon on an ORC/Cdc6 complex (Stillman, 2005). *In vitro*, Cdc6 influences ORC sensitivity to proteases, suggesting an induced structural change (Herbig et al, 1999; Mizushima et al, 2000). Importantly, Cdc6 is phosphorylated and/or exported from the nucleus during S phase to prevent re-initiation of DNA synthesis at replication origins (Alexandrow and Hamlin, 2004).

Cdc6 cooperates with another protein, termed Cdt1, to aid in the licensing of replication origins. Cdt1 physically interacts with Cdc6 and is similarly required for licensing chromatin for replication (Tada et al, 2001). Together, Cdc6 and Cdt1 function with the ORC to load the MCM helicase onto the replication origin, the final stage of replication licensing. To prevent the re-initiation of DNA synthesis at replication origins, Cdt1 is ubiquitinated and degraded after the initiation of DNA replication (Arias and Walter, 2005; Arias and Walter, 2006; Senga et al, 2006). Unique to multicellular eukaryotes, the protein geminin is expressed in S phase where binds to and inactivates Cdt1, thereby also inhibiting the ability of Cdt1 to promote helicase loading at replication origins and thus preventing origin re-firing.

The putative replicative helicase is composed of six proteins termed the MCM (mini-chromosome maintenance) complex. The six subunits (Mcm2-7) are related to one another and have been conserved throughout eukaryotes and archaea. The MCM complex is required not only for the initiation of DNA replication but also for the continued elongation phase of DNA synthesis, as chromatin immunoprecipitation studies from budding yeast and *Xenopus* show that the MCMs move with the replication fork bidirectionally away from the replication origin during S phase (Aparicio et al, 1997; Pacek and Walter, 2004; Pacek et al, 2006). Data from both yeast and *Xenopus* replication systems show that multiple MCM complexes are loaded at each replication origin in a process requiring the ATPase activity of ORC and Cdc6 (Bowers et al, 2004; Edwards et al, 2002; Randell et al, 2006). Although the precise rationale for loading multiple MCMs at each origin is currently unclear, it may give rise to the observation that in mammalian cells replication often initiates throughout broad chromosomal domains (Alexandrow et al, 2002). Once the MCMs have been loaded at the replication origin, the initiation proteins are referred to as a pre-replication complex (pre-RC), and the replication origin is referred to as being licensed, awaiting additional signals to be activated and proceed to the next stage of replication initiation.

Though the general roles of ORC, Cdc6, Cdt1 and the MCMs in pre-RC formation are well established, there have been additional factors more recently identified that appear to affect the establishment of pre-RCs at replication origins. One such factor, Noc3, was identified as a suppressor of an MCM5 mutation in budding yeast, where it also associates with replication origins (Zhang et al, 2002). Elimination of Noc3 impaired the origin binding of Cdc6 and MCM proteins, suggesting a role for Noc3 in pre-RC assembly. Although the precise biochemical function of Noc3 in replication initiation has not been established, its primary role had previously been believed to be in pre-rRNA processing (Milkereit et al, 2001), suggesting a link between ribosome biogenesis and the regulation of DNA replication. A link between ribosome formation and DNA replication was further supported by the identification of Yph1 as an ORC-interacting protein (Du and Stillman, 2002). Yph1 interacts in a large complex with ORC and MCM components, and is essential for both ribosome biogenesis and for entry into S phase. Unique to vertebrate chromosomal replication, a new member of the MCM family, termed Mcm8 (Gozuacik et al, 2003; Johnson et al, 2003), has been identified as functioning at the G1-S phase transition in human cells. Elimination of Mcm8 by RNAi in cultured human cells reduced Cdc6 and MCM binding to chromatin (Volkening and Hoffmann, 2005), suggesting a role for Mcm8 in pre-RC establishment after ORC formation at replication origins.

Although the primary components of pre-RCs are largely conserved throughout eukaryotes, the existence of unique proteins such as MCM8 and geminin implicate a role for novel, metazoan-specific factors that can regulate the initiation of DNA replication. This additional level of regulation may be important for the maintaining the larger genomes in higher eukaryotes and avoiding the potentially more catastrophic effects of genome instability in these organisms.

Activating the replication origin

Origin licensing and pre-RC assembly occur between late mitosis and G1 phase of the cell cycle but are insufficient for DNA synthesis. These complexes are activated at replication origins to undergo replication initiation in a temporal order throughout S phase, and although all the mechanisms controlling this temporal activation remain to be elucidated, it requires the activity of two kinase complexes (Bell and Dutta, 2002). The first is the cyclin-dependent kinase (CDK) activity that plays a well-recognized role in controlling other aspects of cell cycle progression. The second kinase is the Dbf4- or Drf1-dependent Cdc7

kinase (DDK) that with CDKs are believed to target several components of the pre-RC to transition the origin into a pre-initiation state defined by the recruitment of additional initiator proteins, most notably the MCM helicase co-factors Cdc45 and the GINS complex (Takahashi and Walter, 2005). It should be also noted that DNA damage and replication checkpoint signaling involve separate kinases that are believed to target components of the pre-RC assembly to prevent the initiation of DNA replication in the presence of genome destabilizing agents or events, such as double-strand breaks (Cortez et al, 2004; Ishimi et al, 2003; Shechter and Gautier, 2005).

Cdc45 is an essential protein for the initiation of DNA replication in yeast, *Drosophila*, and *Xenopus* interacts with both components of the pre-RC as well as DNA polymerases (Mimura and Takisawa, 1998; Mimura et al, 2000; Zou, Mitchell, and Stillman, 1997; Zou and Stillman, 1998). Cdc45 may serve as a bridge between the proteins that select origin sites with the actual replication machinery. Cdc45 association with replication origins coincides with their time of activation (Aparicio et al, 1999). Similarly, Cdc45 interacts with sites of DNA unwinding on plasmids in *Xenopus* egg extracts (Pacek and Walter, 2004; Pacek et al, 2006) and interfering antibodies against Cdc45 abolish unwinding. In a recent report, helicase activity was detected in a biochemically purified complex containing *Drosophila* Mcm2-7, Cdc45, and the GINS complex (Moyer et al, 2006).

Similar to Cdc45, the GINS complex has been shown in both yeast and *Xenopus* egg extracts to bind replication origins after pre-RC formation and localizes at the site of DNA unwinding (Kubota et al, 2003; Pacek et al, 2006; Takayama et al, 2003). The GINS complex is composed of four subunits (Sld5, Psf1, Psf2, and Psf3) that by electron microscopy shows a ring-like structure around DNA (Kubota et al, 2003) and apparently stimulates the processivity of DNA polymerase epsilon (Seki et al, 2006). Moreover, an interaction of GINS at the replication origin is required for Cdc45 binding to the origin, and also for the stable association of Cdc45 with the MCM helicase (Gambus et al, 2006). Although recent data suggest that MCM2-7, Cdc45, and GINS may form the active replicative helicase (Moyer et al, 2006), additional factors have been described that are also required for activation of replication origins.

Identified in a yeast plasmid maintenance screen along with other members of the MCM2-7 complex, the protein MCM10 associates with the MCM2-7 complex and replication origins in budding yeast and *Xenopus* (Kawasaki et al, 2000) at a step after pre-RC assembly and before recruitment of Cdc45

and unwinding of the replication origin (Ricke and Bielinsky, 2004; Sawyer et al, 2004; Wohlschlegel et al, 2002)). It regulates the stability of the DNA polymerase alpha catalytic subunit (Ricke and Bielinsky, 2004) and thus may also function at later stages of DNA replication. Interestingly, a recent report showed MCM10 to contain primase activity (Fien and Hurwitz, 2006), an activity previously believed to be unique to the DNA polymerase alpha/primase complex.

Identified in budding and fission yeast, as well as in *Xenopus* and human cell systems, a protein termed TopBP1 (also known as Cut5/Dpb11/Mus101) has been demonstrated to function like MCM10, after pre-RC formation and before unwinding of the replication origin (Hashimoto and Takisawa, 2003; Makiniemi et al, 2001; Van Hatten et al, 2002). Its role in DNA replication has been primarily described in *Xenopus*, where TopBP1 functions after CDK and Cdc7 kinase activity and is required for the recruitment of the MCM helicase co-factor Cdc45 onto chromatin. Also similar to MCM10, Mus101 may function outside replication initiation, most noticeably in replication checkpoint signaling (Kumagai et al, 2006; Parrilla-Castellar and Karnitz, 2003).

Two other proteins shown in yeast to be required for Cdc45 recruitment to replication origins include the proteins Sld2 and Sld3, both of which were initially thought to lack homologs in metazoan species. Sld2 was originally shown in budding and fission yeast (Drc1) to be required for chromosomal DNA replication (Kamimura et al, 1998) and to interact with TopBP1/Dbp11/Cut5 (Wang and Elledge, 1999), suggesting an additional role in checkpoint signaling. As with MCM10 and TopBP1, the interaction of Sld2 with replication origins occurs after pre-RC assembly but, interestingly, Sld2 is a target of the CDK activity that is required for origin activation (Masumoto et al, 2002; Noguchi et al, 2002; Tak et al, 2006). Only recently was an Sld2 homolog identified in metazoans, in the form of an N-terminal domain on the RECQL4 helicase mutated in Rothmund-Thomson syndrome (Matsuno et al, 2006; Sangrithi et al, 2005). Although the RECQL4 protein is from a large family of helicases related to the bacterial RecQ helicase, the helicase domain was shown not to be essential for promoting replication in *Xenopus* egg extracts. Interestingly, only the domain similar to the yeast Sld2 protein was required for chromosomal replication in egg extracts, at a step after pre-RC formation and Cdc45 recruitment to chromatin, but before RPA binding to replication origins. These results suggest a role for Sld2 during the time of unwinding at the replication

origin, and indicate an apparent divergence in the regulation of DNA replication initiation in metazoans compared to yeast.

Similar to Sld2, a homolog of the yeast protein Sld3 has not been identified in any metazoan genome. Sld3 binds Cdc45 directly in both budding and fission yeast (Kamimura et al, 2001; Nakajima and Masukata, 2002) and is required for the maintenance of Cdc45 at replication origins. Recent evidence suggests that it interacts only transiently with MCMs at replication origins, leading to origin activation (Kanemaki and Labib, 2006). The lack of an identifiable homolog in metazoans suggests a possible divergence in the regulation of Cdc45 recruitment to replication origins between unicellular and multicellular eukaryotes (Kanemaki and Labib, 2006; Takayama and Kamimura, et al, 2003).

Together these data suggest that although many proteins required for activating replication origins are conserved throughout eukaryotes, there may be unique factors involved in this regulation in higher eukaryotes. Moreover, these novel regulations may take the form of new proteins that are either lacking completely in the yeasts or that are appended to proteins having apparently separate functions from those required only for the initiation of DNA synthesis.

Initiation of DNA synthesis

The CDK- and DDK-dependent recruitment of Cdc45 and GINS to the replication origin coincides with the conversion of the pre-RC to a pre-initiation complex (pre-IC). Demonstrated primarily in *Xenopus* egg extracts, this recruitment also activates the MCM helicase activity to unwind the DNA at the replication origin (Pacek and Walter, 2004; Pacek et al, 2006; Shechter, Ying and Gautier, 2004; Walter, and Newport, 2000). The single-stranded DNA that is generated by DNA unwinding is stabilized by the eukaryotic single-stranded binding protein RPA, providing a template for DNA synthesis. The subsequent recruitment of DNA polymerase alpha/primase allows the synthesis of a short RNA primer and the initiation of DNA synthesis. Interestingly, many of the initial studies of replication initiation and elongation were done using viruses such as SV40 and papillomavirus that infect mammalian cells. Although these viruses use different initiator proteins that recognize the viral replication origin and serve as the helicase that unwinds the origin DNA, host cell factors such as RPA and DNA polymerase alpha/primase are subsequently utilized for DNA synthesis. In all eukaryotic systems, the primed template generated by DNA polymerase alpha/primase is recognized by replication factor C (RFC), which then loads the polymerase

clamp PCNA onto the DNA. PCNA acts as a processivity factor for DNA polymerases delta and epsilon during the elongation phase of DNA replication. Evidence in a variety of eukaryotic replication systems suggests the MCM helicase is also involved in this process as the replication fork moves bidirectionally away from the replication origin.

Replicator elements in eukaryotes

Unicellular replicators

Although a significant number of the proteins involved in initiating DNA synthesis at replication origins are conserved throughout eukaryotes, the particular sequences to which they bind, termed replicators, show no clear level of conservation. In budding yeast, scORC is bound to chromosomal sites containing an ARS (autonomously replicating sequence)-consensus sequence (ACS), an AT-rich DNA element found in a variety of sequences that impart on plasmids the ability to replicate autonomously. Indeed the ORC was originally identified based on its affinity for these specific DNA sequences (Bell and Stillman, 1992). Replication origins characterized in budding yeast generally show similar functional elements, and are typically small (100-200 bp) and usually contain identical sequence elements including an ACS, transcription factor binding sites, and sequences showing intrinsic helical instability (Bell, 2002; Bell and Dutta, 2002; Diffley and Stillman, 1988; Natale et al, 1993). Microarray technology has allowed identification of all ORC binding sites and replication origins in the budding yeast genome (Wyrick et al, 2001), and these data demonstrate a strong correlation between the presence of an ARS element and ORC binding. However not all ARS elements that promote plasmid maintenance also serve as functional replication origins in the chromosome, implicating a role for the local chromatin environment in the regulation of replication initiation. In fission yeast, ARS elements have been identified in plasmid maintenance assays as in budding yeast. Replication origins in fission yeast are typically much larger (500-2000 bp) and contain asymmetric stretches of AT-rich DNA, but show no identical sequence like the ACS in budding yeast (Dai et al, 2005; Segurado et al, 2003; Takahashi et al, 2003). Moreover, in fission yeast, the Orc4 subunit contains an AT-hook motif that apparently promotes its binding to these sequences (Gaczynska et al, 2004; Kong and DePamphilis, 2001). This motif is not found in any other eukaryotic Orc4 homolog, thus suggesting a unique feature of origin recognition in fission yeast. Though yeast

systems have been useful in characterizing the protein necessary for replication initiation, the systems have proved largely inadequate for characterizing and identifying sites of initiation in metazoans.

Embryonic systems

A number of embryonic systems, including extracts prepared from *Xenopus* eggs and *Drosophila* oocyte follicle cells have been critical to understanding the regulation of DNA replication in metazoans. The systems also highlight how DNA replication origins are differentially regulated through development and under specific developmental needs, such as during gene amplification for egg shell production.

***Xenopus* egg extracts**

It has been widely recognized that any DNA substrate, whether it be sperm chromatin or plasmid DNA, can be completely replicated in a cell cycle-regulated manner when incubated in an extract prepared from *Xenopus* eggs (Smythe and Newport, 1991; Tutter and Walter, 2006). This apparent lack of sequence specificity for replication initiation suggested a regulation of origin function clearly different than that in yeast or somatic mammalian cells. It was initially suggested that the great abundance of replication proteins in the extract could account for this lack of sequence specificity, though some level of origin regulation was clearly evident due to the regular spacing of replication origins at about 7-15 kb from one another (Blow et al, 2001; Lemaitre et al, 2005; Walter and Newport, 1997). The lack of site specificity is a function of time in development, as replication from the rDNA locus is random in early embryos but becomes specific to an intergenic spacer region at the mid-blastula transition (Hyrien and Mechali, 1993; Hyrien et al, 1995). Recent data suggest that the organization of chromosomes and chromatin loops is likely to determine the distribution of ORC (Lemaitre et al, 2005), and hence initiation sites, across the genome in early development. Furthermore, the recruitment of transcription factors to specific sites on plasmids could impart replication initiation from that site in the absence of transcription (Danis et al, 2004), arguing that initiation sites may be chosen directly though the interaction of ORC or pre-RC components with transcription factors or though a modification of chromatin structure. Similar effects of transcription factors on DNA replication have been found in yeast and SV40.

Drosophila chorion locus

In Drosophila, the chorion locus in oocyte follicle cells undergoes hundreds of rounds of re-replication to amplify the chorion gene, a process that is necessary for proper egg shell development (Tower, 2004). The best characterized region that is amplified is on chromosome 3, where two DNA sequence elements are necessary and sufficient for replication and amplification. These sequences have been termed ACE3 and oriβ, and are a classic example of a genetic replicator because together the two elements can direct amplification when inserted at other locations in the Drosophila genome (Lu et al, 2001). Mutational analyses demonstrated that the oriβ sequence contained two essential elements, a 140 bp region and a 226 bp AT-rich region likely to encode a DNA unwinding element (Zhang and Tower, 2004). Interestingly the oriβ sequence could not be replaced with a yeast replication origin (ARS1), suggesting that DmORC does not simply recognize the same AT-rich DNA of a yeast replication origin. Rather, recent data suggest that although DmORC shows preference of AT-rich DNA, it is apparently not a typical sequence-specific DNA-binding protein because it bound with similar affinities to both Drosophila replicator sequences as mutant and wild-type ARS elements from budding yeast and even other non-origin DNA sequences. These results suggest that sequence alone is insufficient to direct DmORC to the ACE3 and oriβ origin sequences. Recently it was demonstrated that DmORC actually has a higher affinity (~30-fold) for negatively supercoiled DNA than linear DNA, thus suggesting a mechanism for recruitment of ORC to replication origins in metazoans that does not necessarily require a defined consensus sequence (Remus et al, 2004).

Mammalian replication origins

The shear size of genomes of higher eukaryotic organisms has made understanding genome replication more difficult. With a haploid genome of 3 billion base pairs and observations that replication begins at origins approximately 100 kb apart, 30,000 replication origins are needed to replicate the human genome during S phase. Currently only about two dozen of these have been identified, and only a handful have been studied in detail. Furthermore, no consensus sequence like that in yeast has been found to be shared among the replication origins thus far identified in human cells (Cvetic and Walter, 2005; DePamphilis, 1999; DePamphilis et al, 2006; Gilbert, 2001; Gilbert, 2004). Indeed plasmid transformation screens attempting to find specific autonomously replication sequences found than any DNA sequence of

sufficient length could confer upon that plasmid the ability to be replicated (Heinzel et al, 1991). Just as was observed for recombinant DmORC, recombinant human ORC was unable to discriminate between DNA fragments containing natural origin sequences and control DNA sequences (Vashee et al, 2003). Nevertheless authentic replicators have been described based on the observation that insertion of the replication origin sequence at ectopic sites in the genome imparts the ability of replication to initiation at the new site (reviewed in Aladjem and Fanning, 2004). Furthermore, recent chromatin immunoprecipitation experiments have demonstrated that ORC and other pre-RC components are recruited to specific replication origins and to replicator elements (Abdurashidova et al, 2003; Ghosh et al, 2006; Keller et al, 2002; Kinoshita and Johnson, 2004; Ladenburger et al, 2002). Another characteristic of mammalian replication origins is the observation that the initiation of DNA synthesis is not necessarily restricted to one specific site at a replication origin (Aladjem et al, 2002; Coffman et al, 2006; Gilbert, 2001; Kamath and Leffak, 2001; Tao et al, 2000; Trivedi et al, 1998; Waltz et al, 1996) as described in yeast (Bielinsky and Gerbi, 1999). Rather many replication origins are better to be described as zones of initiation, with the initiation of DNA synthesis occurring at any of several sites within a broad chromosomal locus. The classic example of this type of replication origin is the DHFR locus from hamster cells, where a 55kb domain between the DHFR and 2BE2121 genes contains at least 20 known sites of initiation each used with varying degrees of efficiency (Dijkwel et al, 2002). Examples of the most studied replication origins in human cells are provided in Figure 2 and are discussed in detail below.

Lamin B2

In contrast to the complex hamster DHFR locus, a replication origin was mapped to a ~500 bp region 3' of the lamin B2 gene in human cells by competitive PCR quantitation of nascent DNA (Giacca et al, 1994) (Figure 2). The lamin B2 replication origin more closely resembles replication origins in budding yeast, based both on its small size and the observation that it displays a cell cycle-dependent footprint that is present in G1 but disappears as cells progress into S phase (Abdurashidova et al, 1998). Within this footprint, the initiation site for DNA synthesis was finely mapped to the nucleotide level (Abdurashidova et al, 2000). *In vivo* crosslinking recently demonstrated the binding of pre-RC components at the locus, indicating for the first time that a mammalian origin is bound by the same replication proteins used in other eukaryotic systems and is regulated by the same cell cycle patterns as in yeast (Abdurashidova et al, 2003).

Transfer of the lamin B2 origin sequence to ectopic loci in human and hamster cells directs replication initiation at the new sites, demonstrating that lamin B2 is an authentic mammalian replicator (Paixao et al, 2004).

β-globin

At the human β-globin locus replication was initially thought to begin at a restricted region of the large locus (Kitsberg et al, 1993), although more recent evidence has supported the idea that replication can initiate at several sites throughout the 50 kb locus in human cells (Kamath and Leffak, 2001), mouse cells (Aladjem et al, 2002), and chicken cells (Prioleau et al, 2003). Control of this origin is complex though, and a deletion of a region 50 kb upstream of the origin containing a subdomain termed the locus control region can inhibit the initiation of replication from this locus (Aladjem et al, 1995; Aladjem et al, 1998; Aladjem, 2004; Cimbora et al, 2000). Like the lamin B2 locus, the β-globin origin contains replicator elements that function to initiate replication when inserted at ectopic genomic sites (Aladjem et al, 1998), although in the case of the β-globin origin, it actually contains two independent non-overlapping sequences that can act separately as replicators (Wang et al, 2004). It remains to be determined if and/or where ORC binds at this locus to allow the initiation of DNA synthesis.

c-myc

Initially identified by our laboratory, competitive PCR analyses demonstrated that DNA replication initiated in a ~3.5 kb region upstream of the c-myc gene in human cells (Leffak and James, 1989; Vassilev and Johnson, 1990). Origins of replication have also been found upstream of the c-myc gene in chicken (Phi-van et al, 1998), frog, and mouse cells (Girard-Reydet et al, 2004), suggesting the presence of evolutionarily conserved sequence elements important for replicator function. Additional work demonstrated that there are actually numerous potential sites of initiation throughout the c-myc locus (Trivedi et al, 1998; Waltz et al, 1996), although a 2.4 kb region immediately upstream of the c-myc gene was shown to be a genetic replicator (Liu et al, 2003; Malott and Leffak, 1999), based its ability to impart origin activity when inserted at ectopic chromosomal sites. Moreover, the structure of the chromatin as well as nucleosome positioning within the replicator was maintained when the 2.4-kb region was inserted elsewhere in the genome (Kumar and Leffak, 1991), suggesting the presence of sequences that have the

ability to control chromatin structure. Mutational analyses of the replicator demonstrated the presence of numerous elements important for replicator function (Liu et al, 2003). These include both transcription factor binding sites and a sequence with intrinsic helical instability, similar to replication origins in yeast and other organisms. Similar to results in yeast and *Xenopus*, the recruitment of transcription factors to the replicator promotes origin activity (Ghosh et al, 2004). Recent work has also demonstrated that pre-RC complexes form at the endogenous c-myc origin as well as at the ectopic c-myc replicator (Ghosh et al, 2006). Moreover, the binding of pre-RC proteins to the origin can be affected by changes in histone acetylation or by deletion of specific sequence elements (Ghosh et al, 2006). Together these results suggest that replicator function in mammalian cells is dependent on both chromatin environment and the presence of specific DNA sequences, as in yeast.

A role for novel factors at replicators

Mutational analyses demonstrate the need for specific sequences for replication to initiate at a replicator sequence, and some of these sequences may serve as binding sites for protein not previously demonstrated to be members of the traditional replication initiation machinery. These binding sites and proteins include RIP60 in the DHFR origin (Altman and Fanning, 2001; Altman and Fanning, 2004), DUE-B and transcription factors at the c-myc origin locus (Casper et al, 2005; Ghosh et al, 2004; Ghosh et al, 2006; Liu et al, 2003), Hox proteins in lamin B2 (Stefanovic et al, 2003), and a MYB/p120-containing complex in the chorion replicator (Beall et al, 2002). Although little is known about how these sequences and corresponding binding proteins regulate origin function, or whether they specifically regulate the formation or activation of pre-RCs. Furthermore, many of the proteins and sequences may be unique to the particular origin locus. Recent data from the Dutta laboratory has shown that the simple recruitment of ORC and Cdc6 to a specific site on plasmids, through fusion of the replication proteins to the GAL4 DNA binding domain, is sufficient to recruit all the factors necessary to initiate DNA synthesis at or near that site (Takeda et al, 2005). However these data do not demonstrate whether a similar situation takes place in the chromosome, where the local chromatin environment is likely to affect origin function and a role for novel replication-promoting accessory proteins may function to increase the efficiency of replication origin firing.

A role for chromatin structure and histone acetylation in DNA replication

The chromatin environment can either inhibit or promote the initiation of DNA synthesis. The packaging of eukaryotic DNA into nucelosomes and higher order chromatin structures has long been recognized as restricting the access of replication and transcription factors to their target DNA sequences. During early development in *Xenopus* and Drosophila embryos, there are dramatic changes in replication origin usage that correlate with the onset of zygotic transcription and global chromatin remodeling, with replication changing from apparently random initiations to that of more specific zones of initiation (Hyrien et al, 1995; Maric et al, 1999; Sasaki et al, 1999). In yeast, deletion of a positioned nucleosome at ARS1 abolished Mcm3 but not ORC binding to the replication origin (Lipford and Bell, 2001), suggesting that chromatin organization can affect pre-RC establishment and hence replicator function.

A prominent modification to chromatin is the reversible acetylation of the N-terminal tails of histones, an event thought to affect the level of chromatin compaction (Li et al, 2006; Lowell and Pillus, 1998) and well-recognized as correlating with activation of gene transcription. The general model is that the recruitment of histone acetyltransferse (HAT) activity to a gene regulatory region promotes gene expression, whereas recruitment of a histone deacetylase (HDAC) inhibits or represses gene transcription (Kornberg and Lorch, 1999). Interestingly, a role for histone acetylation in DNA replication was suggested by the observation that the pre-RC components Orc1 and Mcm2 interact with the HAT HBO1 (Burke et al, 2001; Iizuka and Stillman, 1999). Furthermore, the helicase co-factor Cdc45 interacts with the HDAC Rpd3 in yeast (Gavin et al, 2002). Deletion of Rpd3 from budding yeast allowed a number of normally late-firing replication origins to initiate DNA synthesis earlier in S phase (Aparicio et al, 2004; Vogelauer et al, 2002). Moreover, the recruitment of an HAT to a late-firing replication origin in yeast promoted the recruitment of Cdc45 to the origin earlier in S phase and shifted the initiation time to early S phase (Vogelauer et al, 2002). Similar results have been found in Drosophila, where recruitment of a HAT or HDAC to the chorion locus increased or decreased origin activity, respectively (Aggarwal and Calvi, 2004). Similarly, mutation of the HDAC Rpd3 resulted in genome-wide hyperacetylation and Orc2 redistribution. In human cells, treatment with the histone deacetylase inhibitor trichostatin A (TSA) caused late-replicating imprinted genes to be replicated earlier in S phase (Bickmore and Carothers, 1995). Together these results argue for a role in histone acetylation in the regulation of replication origin activity in all eukaryotes.

DNA unwinding elements

The most common type of DNA sequence found at replication origins in bacteria and eukaryotes is AT-rich DNA (Kowalski and Eddy, 1989; Lin and Kowalski, 1994; Natale et al, 1993). Since AT-rich DNA often unwinds with less energy than other sequences, these sequences may function as DNA unwinding elements at replication origins. The principle is that these sequences, due to their intrinsic helical instability, are likely to influence the efficiency of replication initiation by generating accessible template for DNA polymerases to copy or for the MCM helicases to unwind. In budding yeast, easily unwound sequences were shown to be present at replication origins and could be functionally replaced with other DUE sequences (Lin and Kowalski, 1997; Natale et al, 1993). Moreover, ARS mutants with DUEs of greater stability could be rescued by growing yeast at elevated temperatures (Natale and Kowalski, 1993). Similarly, the observation that DNA at the rDNA locus in budding yeast was not as easily unwound *in vivo* as DNA at other replication origins was suggested to be responsible for the inefficiency of replication initiation from the rDNA locus (Miller et al, 1999). Mammalian replication origins typically correspond to intergenic promoter sites, which are similarly known to contain sequences that aid transcription initiation. Together these results suggest that AT-rich DNA at mammalian replication origins may serve as binding sites for ORC or other proteins required for DNA replication initiation.

The metazoan replicator

Although a great deal of effort has been put forth to elucidate replicator structure in metazoans, the lack of a specific consensus sequence for either ORC recruitment or replicator function has hampered the development of a comprehensive model for replication initiation in metazoans. However, studies of individual replication origins clearly demonstrate the requirement for specific DNA sequences in replicator function. Indeed the most common DNA sequence element found among all replication origins is a region of AT-rich DNA, which is often helically unstable and may thus function as a DNA unwinding element. Furthermore, the large DNA content of metazoan genomes and the complex chromatin structures that are required to organize this DNA also suggests a role for chromatin in the regulation of DNA replication initiation. A minimal replication origin in metazoans is therefore likely to involve at least two essential factors. The first is an open and accessible chromatin environment to allow accessibility of replication

initiation and activation proteins to DNA. A second component of metazoan replicators are likely to be DNA sequences that have a higher probability of unwinding and therefore generating accessible template for DNA polymerases. Thus the focus of the work described below is the characterization of two related factors that influence the activity of replication origins, histone acetylation and a newly identified DNA unwinding element binding protein.

MATERIALS AND METHODS

Cell culture

HeLa, HCT116, and H1299 cells were maintained as adherent cultures in DMEM containing 10% newborn calf serum and 10 μg/ml gentamicin (Gibco). The HCT116 cell line expressing reduced levels of Orc2 (Orc2Δ/-) were created in the laboratory of Dr. Anindya Dutta. H1299 cells were generously provided by the laboratory of Dr. Steven Berberich. Daudi cells were obtained from ATCC and maintained as suspension cultures in RPMI 1640 medium with 2 mM L-glutamine, 10% fetal bovine serum, and 10 μg/ml gentamicin. All stable cell lines that were created employed plasmid vectors containing neomycin resistance genes, and selection was performed for 2-3 weeks with 400 to 1000 μg/ml G418 (Gibco).

The insect cell line Sf9 was obtained from Invitrogen and used for expression and purification of recombinant DUE-B proteins. Sf9 cells were maintained in either Grace's insect medium supplemented with yeastolate, lactalbumin, pluronic acid, and 10% fetal bovine serum or in the serum-free medium Sf-900 II SFM (Invitrogen). Both media included the antibiotic gentamicin (10 μg/ml), except during transfections. Cells were grown both as suspension cultures (100 ml to 500 ml) or as adherent cultures.

Cell synchronization

Cells were accumulated in late G1 phase by treating cells for 18 to 24 hours with 200 to 500 μM mimosine (Sigma). Arrest in early S phase was achieved by treating for 20 to 24 hours with 1 μg/ml aphidicolin (Sigma). Cells were also arrested in early S phase by overnight treatment with the ribonucleotide reductase inhibitor hydroxyurea (2 mM HU; Sigma). Mitotic arrest was achieved by incubating cells with 100 ng/ml nocodazole (Sigma) for 14 to 24 hours.

Nascent strand abundance assays

HeLa cells were plated in 15 cm tissue culture plates to be ~70% confluent at time of harvest. Several hours before harvesting cells, a 100 ml 1.25% alkaline agarose gel (50 mM NaOH, 1 mM EDTA) was prepared and stored in alkaline buffer (50 mM NaOH, 1 mM EDTA) at 4°C. Although most assays employed low-melt agarose (SeaPlaque), some assays employed regular agarose (Invitrogen) and yielded similar amounts of nascent DNA. Cells were harvested by trypsinization, washed once with ice cold PBS, then pelleted and resuspended in a minimal volume of 10% glycerol (in PBS). Electrophoresis buffer was

removed from wells and then 60-70 µl of the cell suspension was loaded into individual wells of the alkaline gel and allowed to lyse for 10 minutes at 4°C. DNA ladders (1 kb ladder and ϕX174/HaeIII, Invitrogen) were then loaded and the gel was electrophoresed overnight at 30 to 40V. The gel was subsequently removed from the gel box and the lanes containing markers cut off from the gel with a razor blade and stained for 30 minutes with 1X TAE containing ethidium bromide. The part of the gel containing samples and corresponding to 1- to 2-kb was excised with a new razor blade and purified with a Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The nascent DNA was eluted with 10 mM Tris, pH 8.0 and stored at -20°C). The total amount of 1- to 2-kb nascent DNA isolated was quantitated by OliGreen fluorescence and ranged from 18 to 25 fg per cell in different preparations, consistent with previous determinations (Malott and Leffak, 1999).

To isolate BrdU-labeled nascent DNA, cells were incubated with 50 µM BrdU for 1 hour prior to typsinization. The harvested cells were then loaded on an alkaline agarose gel as describe above, and the short, 1- to 2-kb nascent DNA removed from the gel and purified. The nascent DNA (1.3 µg) was then incubated with 50 µl of a 10X Immunoprecipitation buffer (1 M NaH_2PO_4, pH 7, 1.4 M NaCl, 0.5% TritonX-100) and 5 µg of anti-BrdU antibody (Pharmingen) for 40 minutes at room temperature. rProtein G agarose (20 µl; Upstate) was added to the immunoprecipitation and incubated for 1 hour at room temperature. After centrifugation, the pelleted beads were incubated overnight at 37°C in digestion buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS, 250 µg/ml proteinase K). After phenol-chloroform extraction the nascent DNA was precipitated in ethanol overnight at -20°C, washed with 70% ethanol, and finally dried and resuspended in 10 mM Tris pH 8. Both the input and immunopreciptated DNA were quantitated by OliGreen fluorescence and then analyzed by Q-PCR.

Preparation of formaldehyde-crosslinked chromatin

To prepare formaldehyde-crosslinked chromatin, the medium from one 15 cm plate of HeLa cells (~80% confluent) was removed and the cells washed twice in cold PBS containing protease inhibitors (PIC, 1 mM PMSF, 1 µg/ml pepstatin A, 1 µg/ml aprotinin). The cells were then scraped from each plate into 5 ml cold PBS plus protease inhibitors (PBS+PIC) and then centrifuged at 2000 rpm for 5 minutes at 4°C. The cells were then resuspended in 1 ml of PBS+PIC and an aliquot taken for cell counting on a hemocytometer. Twenty percent of the cell suspension was taken for preparation of whole cell extracts by

sonication of the cell pellet in 1X SDS-PAGE sample buffer. The remaining cells (80%) were centrifuged for 5 minutes at 4000 rpm in a swinging bucket rotor using a Sorvall Biofuge at 4°C. Cells were resuspended in 1.34 ml cold hypotonic Buffer A (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, PIC). Triton X-100 was added to a final concentration of 0.04% and the cells incubated on ice for 10 minutes. The nuclei were subsequently pelleted by centrifugation for 5 minutes at 4000 rpm at 4°C. Nuclei were washed once in Buffer A without Triton X-100, re-centrifuged, and then resuspended in 133 µl Buffer A. A prewarmed solution of Buffer A containing 1% formaldehyde (1.2 ml) was added to the cell suspension and then incubated for 10 minutes at 37°C. Cross-linked nuclei were then pelleted and washed twice with PBS plus 0.5% NP-40. Next the nuclei were resuspended in 107 µl Buffer B (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease inhibitors) and a 10% sarcosyl solution added to a final concentration of 2%. Cells were placed for 5 minutes on ice and then layered over a 0.5 ml sucrose cushion made in Buffer B. After centrifugation for 5 minutes at 4000 rpm at 4°C the chromatin pellet was resuspended 1X SDS-PAGE sample buffer, heated for 10 minutes at 95°C, and finally sonicated. In experiments where the chromatin was to be used for immunoprecipitation and purification of DNA for Q-PCR analysis, the chromatin was instead resuspended in TE, as described (Ghosh et al, 2006). Experiments in which the cross-linked chromatin was used for ChIP analyses were performed by Dr. Maloy Ghosh. The use of these data is gratefully acknowledged.

Quantitative PCR

Quantitative, real-time PCR (Q-PCR) analyses were performed on the Applied Biosystems GeneAmp 5700 and Prism 7000 sequence detection systems using SYBR Green fluorescence to monitor amplification. Copy numbers were determined by comparison to standard curves generated for each primer set with known amounts of sheared, genomic DNA.

Random sequence primer PCR

Amplification of nascent DNA with random sequence 10-mer primers (Table 2) was performed in reactions containing 200 µM dNTPs, 2.5 U Taq polymerase (Qiagen), 50 mM KCl, 2 mM $MgCl_2$, 60 ng total primer, and 5 ng nascent DNA. The PCR amplification was performed with 45 cycles of 94°C for 30

seconds, 35°C for 1 min, and 72°C for 2 minutes. The PCR products were purified (Qiagen), electrophoresed on 2% agarose gels, and stained with ethidium bromide to visualize the DNA.

Western blotting

Protein expression in cell lysates and egg extracts was determined by SDS-PAGE and western blot analysis. Anti-actin antibody was purchased from Oncogene. Anti-acetylated histone H4 antibodies were from Upstate Biotechnology. Polyclonal antibodies against human ORC1, Orc2, Mcm3 and MCM7 used in ChIP assays and some western blots were obtained from Dr. Aloys Schepers. Anti-DUE-B antibody was raised in rabbits against a 6xHis-tagged recombinant human DUE-B expressed in bacteria. Anti-xOrc2 antibody was raised in rabbits against baculovirus-expressed recombinant xOrc2. Both the anti-DUE-B and anti-xOrc2 antibodies have been previously described (Casper 2004). Rabbit anti-ORC1 antibody was generously provided by Dr. Chikashi Obuse (Kyoto University; Tatsumi et al, JBC 2003 I). Anti-MEK2 antibody was purchased from LabVision Corporation. Anti-RPA70, anti-Cdc6, anti-Rad9, anti-p21, and anti-Cdc45 antibodies were purchased from Santa Cruz Biotechnology. Anti-PCNA, anti-cdk1, anti-cdk2, anti-cdk4, anti-cyclin A, anti-cyclin D, anti-p19, anti-Rb2 antibodies were from Transduction Laboratories. The monoclonal anti-xDnmt antibody 4A8 used to detect xDnmt1 in *Xenopus* egg extracts was the gift of Dr. Shoji Tajima (Osaka University) (Shi et al, 2001). Antibodies against human Dnmt1 were obtained from Santa Cruz Biotechnology (sc-20701) or Dr. William Nelson (Johns Hopkins University) (De Marzo et al, 1999). Anti-His (C-term) monoclonal antibody was purchased from Invitrogen. The anti-phosphoserine antibody Q5 was obtained from Qiagen. Dr. Andrew Vaillant at REPLICor Incorporated (Laval, Quebec) provided anti-Ku80 antibody. Antibodies against the *Xenopus* proteins Mus101/Cut5, DNA polymerase alpha catalytic subunit, and the Bloom's syndrome helicase were provided by Dr. Matt Michael (Harvard University), Dr. Shou Waga (Osaka University), and Dr. Hong Yan (Fox Chase Cancer Center), respectively.

Flow cytometry

For analysis of DNA content, 0.5 to 1 x 10^6 cells harvested by trypsinization, washed in PBS, and then fixed in 70% ethanol at -20°C typically overnight. Cells were rehydrated in PBS and then resuspended in PBS containing RNase A (30-100 U) for 20 minutes at 37°C. Propidium idodide (Sigma) was then added

to the cell suspension to a final concentration of 50 μg/ml and the cells incubated at 4°C for 30 minutes. Finally the samples were warmed to room temperature and analyzed on a Becton Dickenson FACScan flow cytometer.

An accurate measurement of S phase cells was performed by incubating cells for 30-60 minutes with 50 μM bromodeoxyuridine (BrdU) prior to trypsinization and fixation. The cells were subsequently incubated under acidic conditions for 10 minutes to denature the DNA. After neutralization with sodium borate, samples were blocked in BSA and incubated with an anti-BrdU antibody conjugated with AlexaFluor-488 (Molecular Probes). Samples were processed as above to stain DNA.

To determine the effect of the histone deacetylase inhibitor TSA on chromatin compaction, cells were stained with the DNA intercalating dye acridine orange, as previously described (Darzynkiewicz et al, 1977b; Darzynkiewicz, 1990). The metachromatic nature of acridine orange is due to its ability to fluoresce green when binding to dsDNA and its characteristic red luminescence when it binds to and precipitates on ssDNA. Because the ability to denature DNA under acidic conditions is influenced by its interaction with histones and other proteins in chromatin (Darzynkiewicz et al, 1977a; Darzynkiewicz et al, 1977b), acridine orange has been useful for analyzing changes in chromatin structure (Darzynkiewicz and Carter, 1989; Darzynkiewicz, 1990). Briefly, HeLa cells left untreated or treated for 4 hours with 100 ng/ml TSA were harvested by trypsinization, washed in PBS, and then fixed overnight in 70% ethanol at -20°C. After rehydration in PBS, cells were treated with 300 U RNase for 20 minutes at 37°C. A 2.5-fold volume of 0.1 M HCl was added to the cell suspension, and after 30 seconds a 10-fold volume of AO staining buffer (90 mM citric acid, 20 mM Na_2HPO_4, and 6 μg/ml acridine orange) was added to neutralize the sample and stain the DNA. Samples were analyzed using a FACScan flow cytometer (Becton Dickenson).

Cell Fractionation

To monitor the regulated binding of replication proteins to chromatin, a cell fractionation method was employed that has been previously used to examine replication and repair protein binding to chromatin *in vivo* (Mendez and Stillman, 2000). Briefly, cells grown in 15 cm culture dishes were washed with cold PBS and then scraped from the plate into PBS containing protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 μg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride). A portion of the cell suspension (20%) was taken for preparation of whole cell extracts by sonication in 1X SDS-PAGE sample

buffer using a Model 500 Ultrasonic Dismembranator (Fisher Scientific; 2-3 minutes, 5 sec on, 5 sec off, 40% amplitude). The remaining cell suspension was pelleted by centrifugation (1300 x g, 4 min, 4°C) and resuspended in ice-cold Buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1mM DTT, protease inhibitors). Some experiments included the addition of the phosphatase inhibitors sodium fluoride (10 mM) and sodium orthovanadate (1 mM) or the HALT phosphatase inhibitor cocktail (Pierce), though no reproducible, observable differences in protein mobility were observed by western blot analysis. To prepare nuclei, Triton X-100 (0.05%) was added and the cells incubated for 10 minutes on ice with occasional inversion of the sample tube. Nuclei were collected by low-speed centrifugation (1300 x g, 4 min, 4°C, Sorvall Biofuge swinging bucket rotor). The S1 supernatant was further clarified by high-speed centrifugation (16,000 x g, 15 min, 4°C) to remove cell debris. This S2 supernatant corresponds to soluble, cytoplasmic proteins. The collected nuclei were washed once in Buffer A without Triton X-100, recentrifuged, then lysed for 30 minutes on ice with Buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitors). Insoluble chromatin was centrifuged (1700 x g, 4 min, 4°C) and washed once in Buffer B before sonication in 1X SDS-PAGE sample buffer.

EMSA

A 123 bp fragment containing the c-myc DUE sequence was amplified from the plasmid pFRT.myc2.4 and labeled by PCR using α-[^{32}P]-dCTP and purified (Qiagen). The primer sequences employed are listed in Appendix A (EMSA-DUE Upper and EMSA-DUE Lower). Binding reactions took place for 15-20 minutes at room temperature and included 25 pmol of the labeled DNA and 25 fmol of rDUE-B in 0.5X TBE, 50 mM NaCl, and 1X blue/orange loading dye (Promega). Reactions were electrophoresed in 0.5X TBE on a 4% native PAGE (pre-run for 30 minutes at room temperature) at room temperature for 50 minutes at 100V. The gel was then dried and analyzed by autoradiography.

Transfections – siRNA/DNA

Short, 21 nt-interfering RNAs (siRNA) were designed by Dr. Andrew Valliant at REPLICor Incorporated (Laval, Quebec) and transfected into human cells using Oligofectamine transfection reagent (Invitrogen) as recommended by the manufacturer. A list of the siRNAs used and their targets are provided in Table 2. The amount of siRNA used often varied from that recommended by Invitrogen. Other

experiments employed the transfection reagent Lipofectamine 2000 (Invitrogen) and gave similar protein knockdown results using the recommended protocol. Transfections usually occurred with cells plated in 6-well plates in medium with serum by without antibiotics. Some siRNA transfection experiments were scaled up for use in larger plates, for example in the case of chromatin isolation, where 15 cm plates were used for transfection.

Transfection of plasmid DNA employed Lipofectamine 2000 (Invitrogen), exactly as recommended by the manufacturer.

Cloning of DUE-B for expression in human cells

Sequencing results indicated that the human DUE-B sequence previously cloned into a variety of expression and transfer vectors in the laboratory contained a mutation that changed asparagine-51 to serine. Thus the correct DUE-B sequence encoding the full-length DUE-B protein (209 amino acids) was amplified from the yeast one-hybrid expression vector pGK16B using primers DUE-BtoBlueBac-Upper and DUE-BtoBlueBac-Lower (Appendix A). Sequencing of the PCR product revealed the correct DUE-B sequence. The PCR product was digested with BamHI and AgeI and then ligated into the mammalian expression vector pcDNA3.1/DUE-B-V5-His (N51S, provided by Dr. John Casper) after removal of the mutant DUE-B cDNA by digestion with BamHI and AgeI. The insertion of the DUE-B sequence placed it in frame with the encoded 6xHis-tag in the vector backbone. The newly constructed plasmid was termed pcDNA3.1/DUE-B-His.

To construct a form of DUE-B resistant to degradation by DUE-B siRNA#2, pcDNA3.1/DUE-B-His was used as template in two PCRs. The first reaction used primers DUE-BtoBlueBac-Upper and siRNA_Mutant-Lower to generate an N-terminal (5'-) DUE-B cDNA where the DUE-B siRNA#2 target sequence was mutated from 5'-AAGCACTGGTCGAAGAGTGTG-3' to 5'-AAACATTGGAGGAAATCCGTC. Both regions code for the protein sequence K-H-W-S-K-S-V, and thus the nucleotide mutations did not affect the translated amino acid sequence. A second PCR using primers DUE-BtoBlueBac-Lower and siRNA_Mutant-Upper generated a C-terminal (3'-) fragment with the identical mutation to the siRNA target side. The two PCR products were then combined in a new PCR with primers DUE-BtoBlueBac-Upper and DUE-BtoBlueBac-Lower to generate a full-length DUE-B cDNA with the mutant, siRNA-resistant sequence. The PCR product was digested with HindIII and AgeI

and cloned into pcDNA3.1, as described above, generating a new construct termed pcDNA3.1/DUE-B-His/siRNA-Res.

Two mutant forms of DUE-B were prepared for expression in human cells, one with a mutation of threonine-81 to an alanine (T81A), and one with a truncation of the C-terminal 62 amino acids (ΔCT). To generate the mutant DUE-B cDNAs for cloning into the mammalian expression vector pcDNA3.1, the plasmid pcDNA3.1/DUE-B-His/siRNA-Res was used as a template for PCR. Mutation of threonine-81 to alanine was done using two PCRs, one in which Thr-81 was mutated in an N-terminal portion of DUE-B with primers DUE-BtoBlueBac-Upper and Thr81Ala-Lower (Appendix A), and a second reaction in which Thr-81 was mutated with primers Thr81Ala-Upper and DUE-BtoBlueBac-Lower (Appendix A). The two PCR products were combined as templates for another PCR in which DUE-BtoBlueBac-Upper and DUE-BtoBlueBac-Lower were used, generating a full-length, human DUE-B cDNA with threonine-81 mutated to alanine. The ΔCT cDNA form of DUE-B was generated by PCR using primers DUE-BtoBlueBac-Upper and Cterm62aa-Lower. The mutant DUE-B cDNA PCR products were digested as above and cloned into pcDNA3.1 for expression in human cells. The two plasmids are referred to as pcDNA3.1/DUE-B-His/T81A and pcDNA3.1/DUE-B-His/ΔCT.

Generation of recombinant DUE-B baculoviruses

The human DUE-B sequence was amplified from pGK16B using primers DUE-BtoBlueBac-Upper and DUE-BtoBlueBac-Lower (Appendix A), then digested with BamHI and AgeI and ligated into the baculovirus transfer vector pBlueBac4.5/V5-His that had also been digested with the same restriction enzymes. The insertion removes the V5 epitope encoded in the plasmid and places the DUE-B sequence in frame with the 6xHis tag. This plasmid, pBlueBac4.5/DUE-B-His was co-transfected with linearized Bac-N-Blue™ DNA (Invitrogen) using Cellfectin reagent into Sf9 cells to generate recombinant baculovirus, as recommended by the manufacturer.

Two mutant forms of recombinant DUE-B were prepared, one in which threonine-81 was mutated to an alanine, and another in which the C-terminal 62 amino acids were deleted. The PCR products were generated as described above for insertion of the mutant DUE-B cDNAs into the mammalian expression vector. The PCR products were similarly digested with BamHI and AgeI and ligated into pBlueBac4.5/V5-His to generate plasmids pBlueBac/DUE-B-His/T81A and pBlueBac/DUE-B-His/ΔCT. The plasmids were

then co-transfected with linearized viral DNA for generation of recombinant baculovirus in Sf9 cells, as described by the manufacturer (Invitrogen).

Individual plaques were screened for proper recombination after 5-7 days on plates containing the chromogenic substrate X-gal. Plaques were screened by PCR to check for purity and the presence of the correct insert into the recombinant virus. High-titer baculovirus stocks were prepared for infection into Sf9 cells grown as either suspension or adherent cultures. An MOI of 10 was typically used for infection of suspension cultures, and an MOI of 5 was used for adherent cultures. Forty-eight hours after infection cells were harvested, washed in PBS, and pellets stored at -70°C until ready for lysis.

Purification of baculovirus-expressed rDUE-B

The cell pellets were lysed with occasional mixing on ice in Lysis Buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.5-1% NP-40, and a protease inhibitor cocktail for 6xHis-tagged proteins (Sigma)). The lysate was clarified by high-speed centrifugation at 10,000 x g for 10-15 minutes at 4°C. The supernatant was then incubated with Ni-NTA agarose (Qiagen) for 2-14 hours, pelleted, and washed with once Lysis Buffer. The agarose beads were then either loaded into a 1 ml polypropylene column (Qiagen) or 0.5 ml Handee spin column (Pierce), depending on the scale of purification. The Ni-NTA agarose beads were then washed 3 times with Wash Buffer-20 (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.1% NP-40, protease inhibitor cocktail) and 0-2 times with Wash Buffer-40 (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 40 mM imidazole, 0.1% NP-40, protease inhibitor cocktail). The rDUE-B was then eluted several times with Elution Buffer (50 mM NaH_2PO_4, pH 8.0, 100 mM NaCl, 200 mM imidazole, protease inhibitor cocktail). The rDUE-B was then concentrated and the buffer exchanged with Protein Storage Buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 10% glycerol) using Amicon Ultra or Microcon centrifugal filter devices (Millipore), depending on the scale of protein to be purified and the level of concentration desired. Appendix B shows a representative image of the purified proteins electrophoresed on SDS-PAGE and stained with Coomassie.

Purification of rDUE-B^{HeLa}

Recombinant DUE-B was also purified from a HeLa cell line stably expressing a 6xHis-tagged DUE-B cDNA using a similar purification procedure. The cell line was generated by transfection of

pcDNA3.1/DUE-B-His into HeLa cells using Lipofectamine 2000 (Invitrogen) and selection for 2 weeks with G418 (Invitrogen). Cells were plated into 96 wells plates at ~1 cell per well and individual clones selected for high-level of expression of 6xHis-tagged DUE-B. Western blot analysis indicated the cell line CloneA1 expressed 6xHis-tagged DUE-B at high level, and this line was used for purification of rDUE-B^{HeLa} and also used in other experiments. For purification purposes, CloneA1 cells were typically grown in 10-15 large culture plates (15 cm) until they were ~80% confluent. The cells were lysed and the protein purified as described above for baculovirus-expressed rDUE-B. Typically 20 to 30 μg of rDUE-B^{HeLa} could be obtained from 10-15 plates of cells.

Preparation of *Xenopus* egg extracts

Female *Xenopus laevis* were maintained according to protocols approved by the Wright State University Laboratory Animal Care and Use Committee (LACUC). Frogs were primed for ovulation by injection with 100 U of pregnant mare serum gonadotropin (PMSG; Sigma) 3-8 days prior to use. To induce egg laying, frogs were injected with 500-800 U of human chorionic gonadotropin (HCG; Sigma) 16-22 hours before the eggs were to be harvested. Frogs induced to lay eggs were placed into individual tanks containing at least 4 liters of either 100 mM NaCl or High Salt Barth solution (110 mM NaCl, 15 mM Tris, pH 7.6, 2 mM KCl, 1 mM $MgCl_2$) to inhibit premature egg activation. Laid eggs were collected in 250 ml beakers, and any activated or sick-looking eggs removed with a Pasteur pipette.

Several different types of egg extracts can be prepared for a variety of *in vitro* assays, and are summarized in Appendix C. The first is the simplest to prepare, termed a low-speed extract or supernatant (LSS), and contains both cytoplasmic proteins and membrane vesicles required to form functional nuclei. The second extract, often referred to as a high-speed extract, separates the cytosol (HSS) from membranes (M). This extract system is useful for studying events that occur on DNA and chromatin that do not require the formation of nuclei. For example, the wrapping of DNA around histones and the formation of pre-replication complexes (pre-RCs) at replication origins is independent of cyclin-dependent kinase (CDK) activity, and thus HSS can be employed for such purposes. In contrast, activation of pre-RCs to allow for the initiation of DNA replication requires the CDK activity, and this activity is concentrated into nuclei that form after the addition of the M fraction to HSS containing DNA. Lastly, a nucleoplasmic extract (NPE) system has been developed that bypasses the need for the formation of nuclei, based on the ability to purify

the concentrated kinase activity found in functional nuclei. This system, not employed here due to a lack of resources, allows sperm chromatin or DNA pre-incubated in HSS to then be added to NPE to allow replication to occur, all in the absence of forming nuclei.

To prepare low-speed egg extracts according to a protocol provided by Dr. Julian Blow, the collected eggs were dejellied for 5-10 minutes in 2.2% cysteine monohydrate HCl, pH 7.8, 1 mM EGTA. Eggs were then gently washed three times in Barth solution (88 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 15 mM Tris, pH 7.4, 0.5 mM $CaCl_2$). Eggs were next activated by adding the calcium ionophore A23187 (2 µg/ml) to the eggs in ~50 ml of Barth solution. After a period of 7-10 minutes the eggs were washed three times with Barth solution, then 4-5 times in cold Extraction Buffer (50 mM KCl, 50 mM HEPES-KOH, pH 7.6, 5 mM $MgCl_2$, 2 mM DTT). Eggs were subsequently transferred to an appropriately sized tube and centrifuged at 800 x g for 1 minute at 4°C to pack the eggs in the tube. Excess buffer was removed from the tubes, and then the tubes were centrifuged in a swinging bucket rotor at 10,000 x g for 10 minutes to spin-crush the eggs. This separates the eggs into a grey insoluble pellet, a brownish cytoplasmic layer, and a yellow lipid plug. The cytoplasm was removed by inserting a needle into the side of the tube and withdrawing the cytoplasmic extract with a syringe. The extract was then transferred to a new tube and supplemented with 10 µg/ml cytochalasin B and diluted 15% (by volume) with Extract Dilution Buffer (50 mM KCl, 50 mM HEPES-KOH, pH 7.6, 0.4 mM $MgCl_2$, 2 mM DTT, 0.4 mM EGTA, 10% sucrose, and 10 µg/ml each of pepstatin A, leupeptin, aprotinin). The extract was then further cleared by centrifugation at 48,500 x g for 20 minutes at 4°C. This spin gives (from bottom to top) a small black insoluble pellet, a small clear gelatinous protein/ribosome layer, a larger loose brown membranous layer, a clear golden cytoplasmic layer, a layer of white membranous material, and a small yellow lipid plug. The golden cytoplasmic layer and white membranous layer on top were removed as described above, supplemented with glycerol to 1%, and frozen as 100 µl aliquots in liquid nitrogen. The extracts were finally stored at -80°C until ready for use.

The second type of egg extract employed is termed a high-speed extract based on the ability to separate the cytoplasmic extract from membrane vesicles. In this protocol, laid eggs are dejellied in 2.2% cysteine describe above, washed three times with 0.25X MMR (1X MMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES, pH 7.8) at room temperature. Eggs are then

washed twice with freshly-made ELB (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM HEPES-KOH, pH 7.6, 50 μg/ml cycloheximide, 1 mM DTT). Eggs are transferred to a tube for centrifugation, with an initial spin at 800 x g to pack the eggs and allow for the removal of excess buffer. Aprotinin, leupeptin, and cytochalasin B are added and the eggs are crushed by spinning at 4°C in a swinging bucket rotor (10,000 x g, 10 min). In this protocol, eggs become activated to move into interphase during centrifugation, and not by incubation with a calcium ionophore as in the previously described method. After centrifugation, the low-speed cytosolic extract layer is removed with a needle and syringe. This low-speed extract can also be used for a variety of *in vitro* assays, or can be spun again at 55,000 rpm for 2 hours to further fractionate the extract into a clear cytosolic layer and an underlying membrane layer. The cytoplasmic layer is removed with a Pasteur pipet and recentrifuged at 150,000 x g for 30 minutes at 4°C, then frozen in liquid nitrogen. The membrane layer is removed with a Pasteur pipet and washed with five volumes of ELB containing 10 μg/ml aprotinin and leupeptin and 5 μg/ml cytochalasin B. The solution is mixed will by inversion and incubated on ice for 10-15 minutes, then layered over ELB containing 0.5 M sucrose. The membranes are then centrifuged at 22,000 rpm for 20 minutes at 4°C and the pelleted membranes frozen as aliquots 0.1 volume that of the high-speed cytoplasmic extract.

Prior to use of the extracts in replication or sperm chromatin binding assays, the extract was supplemented with freshly added protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A). Cycloheximide (250 μg/ml) was added to extracts prepared in its absence to inhibit protein synthesis and limit DNA replication to one round. Nocodazole (3 μg/ml) was typically added to also prevent movement into another S phase. Lastly, an ATP regeneration system (2 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine phosphokinase) was added to maintain ATP levels.

Immunodepletion of proteins from *Xenopus* egg extracts

To deplete proteins from *Xenopus* egg extracts, antibodies against the protein to be depleted were coupled to recombinant Protein G agarose beads. After several washes with ELB to remove any unbound antibody, the antibody-bound beads were incubated with egg extract for a period of 20-30 minutes at 4°C. The beads were then pelleted by microcentrifugation (5000 rpm, 2 min, 4°C) and the supernatant transferred to a new tube containing antibody-bound agarose beads and the process repeated. Typically 3-4 rounds of immunodepletion were required to deplete xDUE-B and xOrc2 by greater than 99% from the

extract. Both low-speed (LSS) and high-speed (HSS) extracts have been used for immunodepletion, with similar efficiencies. Due to previous results suggesting xDUE-B may co-fractionate with the membrane fraction, membranes were incubated with the HSS for immunodepletion. An aliquot of the extract after each round of depletion was typically checked by western blot to monitor the extent of immunodepletion.

***Xenopus* sperm chromatin replication assay**

To monitor DNA synthesis in *Xenopus* egg extracts, LSS or HSS+M was supplemented with α-[^{32}P]-dCTP (0.1 μCi/μl) and sperm chromatin was added to a final concentration ranging from 1,000 to 10,000 sperm per μl of extract (typically 2000-3000 sperm/μl). Reactions (5 μl) were incubated for 30 to 120 minutes at room temperature before stopping with 8.3 μl of Stop buffer (8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% bromophenol blue, 80 mM Tris pH 8). After all the time points were collected, proteinase K (40 μg) was added to each sample and then incubated at 37°C for at least 1 hour. In some experiments, the reactions were extracted with phenol-chloroform and precipitated with ethanol before continuing with the analysis. Samples were then loaded directly into wells of a 0.8% 1X TAE agarose gel and electrophoresed. The gel was subsequently dried and exposed to film.

Sperm chromatin spin-down experiments

To detect the binding of replication factors to chromatin, replication reactions (20-25 μl) were prepared as described above (without α-[^{32}P]-dCTP) and incubated for the desired time at room temperature. Reactions were then stopped by addition of a 10-fold volume of ice-cold buffer XB (10 mM HEPES, pH 7.6, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5% sucrose, 1 mM DTT, protease inhibitors) containing 0.05% Triton X-100 and incubated for 2 minutes on ice. Chromatin was layered over a 0.7 M sucrose cushion (1 ml) made in buffer XB and centrifuged in a swinging bucket rotor (5 minutes, 3900 x g, 4°C) in a Sorvall Biofuge. The chromatin pellets were washed once in buffer XB and recentrifuged, then boiled in 2X SDS-PAGE sample buffer and separated by SDS-PAGE and analyzed by immunoblot.

Limited proteolysis

Limited proteolytic digestion of rDUE-B performed using the proteases trypsin, chymotrypsin, and V8 protease or chemically by treatment with formic acid (Sigma). Trypsin digestions were done at 37°C at a ratio (w:w) of 1:20 to 1:400 (trypsin:DUE-B) for up to 18 hours in either 25 mM ammonium

bicarbonate (pH 8.5) or in 100 mM Tris (pH 8.5). Digestion with chymotrypsin at a w:w ratio of 1:50 in 100 mM Tris, pH 7.8, 10 mM $CaCl_2$ at 30°C for up to 18 hr. V8 protease digestion was done at 1:20 (w:w) in 100 mM Tris, pH 7.8 at 37°C for 2 hrs. Digestion with 70% (final concentration) formic acid was done at 50°C for 16 hrs, as previously described (Piszkiewicz et al, BBRC 1970). The samples were then dried under vacuum and redissolved in 100 mM Tris, pH 8.5, for SDS-PAGE.

For proteolysis experiments in the presence of DNA, DUE-B (10 pmol) was digested with trypsin (1:200 to 1:400, trypsin:DUE-B) after a 10-15 minute pre-incubation with either a 123 bp PCR product (100 pmol) containing the c-myc DUE (primers in Appendix A) or with 54 nt unannealed or 54 bp annealed oligonucleotides (20:1 DNA:DUE-B) corresponding to the c-myc DUE region (Appendix A). Alternatively 100 ng of poly-dIdC/poly-dIdC was pre-incubated for 15 minutes with 200 ng rDUE-B prior to addition of trypsin (1:160) and incubation at 37°C for 2 hours.

The digestion reactions were stopped by addition of SDS-PAGE sample buffer, then separated by SDS-PAGE and analyzed by either silver staining or western blot, as indicated.

D-amino acid deacylase assay

Aminoacylation of *E. coli* tRNA was performed similar as described (Soutourina et al, 2000) to minimize contamination by L-aspartic acid. A 500 µl reaction containing 100 mM Tris, pH 7.6, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 2.5 mM ATP, 1% glycerol, 0.6 mM β-mercaptoethanol, 5 µM [^{3}H]-D-aspartic acid (Perkin Elmer), 25 µM *E. coli* tRNA (Sigma), and 1250 U mixed *E. coli* tRNA synthetases (Sigma) was incubated for 10 minutes at 37°C. After overnight precipitation with ethanol at -20°C, the supernatant was lyophilized, resuspended in water, and further used in an aminoacylation reaction at 37°C for 90 minutes with an additional 12.5 nmol *E. coli* tRNA and 1250 U of *E. coli* tRNA synthetase. The reaction was then extracted with phenol-chloroform, precipitated with ethanol, and used fresh in deacylation reactions.

E. coli tRNA (Sigma) aminoacylated with [^{3}H]-D-asp was incubated in a 50 µl reaction containing 20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, and 500 fmol of the indicated form of recombinant DUE-B from baculovirus-infected insect cells. The reaction was incubated at 37°C for 10 minutes before addition of glycogen and precipitation with ethanol at -20°C overnight. After centrifugation the supernatant was analyzed by liquid scintillation counting.

ATPase assay

Reactions containing 25 mM Tris, pH 7.6, 1 mM DTT, 0.2 mg/ml BSA, 5 mM $MgCl_2$, 100 μM unlabeled ATP, 100 nM γ-[^{32}P]-ATP (Perkin Elmer) and the indicated amount of recombinant DUE-B were incubated at 37°C for 60 minutes. The reactions were then stopped by addition of EDTA to 50 mM and frozen in liquid nitrogen. An aliquot of each reaction was then spotted on a PEI-Cellulose TLC plate (Selecto Scientific) that was developed in 0.8 M acetic acid/0.8 M LiCl and then analyzed on a phosphorimager.

Computational algorithms

The program WEB-THERMODYN (Huang and Kowalski, 2003) was used to estimate the free energy of unwinding of DNA sequences at the c-myc replication origin, and is available at the following address: http://wings.buffalo.edu/gsa/dna/dk/WEBTHERMODYN. WEB-THERMODYN uses a nearest-neighbor algorithm to compute the helical stability of double-stranded DNA sequences. PONDR (http://www.pondr.com) was used to determine regions of the DUE-B protein likely to be disordered, based on amino acid sequence, hydropathy, and protein flexibility (Liu et al, 2006; Romero et al, 2001; Romero et al, 2004). The program DEPP (Disorder Enhanced Phosphorylation Predictor) was used to predict the likelihood of phosphorylation at sites throughout DUE-B and can be found at the PONDR website.

Mass spectrometry

MALDI-TOF and tandem LC-MS/MS mass spectrometry analyses were performed at either the Ohio State University Mass Spectrometry and Proteomics Facility or the Genome Research Institute (Cincinnati, Ohio). Proteins were submitted either in-gel or in solution for analysis.

DUE-B crystallization

Recombinant DUE-B from baculovirus-infected insect cells, containing a 6xHis-tag on the C-terminus of the protein, was provided to Dr. Satish Nair (University of Illinois-Champaign/Urbanna) for preparation of crystals suitable for x-ray diffraction. Details of the procedure used for crystallography are described (Kemp et al, manuscript in preparation).

RESULTS

I. The histone deacetylase inhibitor trichostatin modulates replication origin activity in human cells

Histone acetylation at replication origins

The lack of a consensus sequence for ORC binding and replication initiation in metazoans implies a more complex regulation of the initiation of chromosomal DNA synthesis than that found in yeast. The larger and more complex architecture of genomes in higher eukaryotes also suggests that this control may involve aspects of chromatin structure, as has been previously described for the regulation of transcription. Promoter elements are widely recognized as being affected by their chromatin environment, and furthermore are known to be specifically affected by changes in histone acetylation, a reversible posttranslational modification on the N-terminal histone tails. The enzymes that acetylate histones are termed histone acetyltransferases (HATs) and the proteins that remove the acetyl group are called histone deacetylases (HDACs). To determine whether the acetylation state of histones is correlated with initiation site selection at previously described replication origin loci, chromatin immunoprecipitation (ChIP) and nascent strand abundance assays were performed at the c-myc, lamin B2, and β-globin replication origins. Although the three replication origin loci vary in size and in degree of initiation site selectivity, all have been shown to contain at least one replicator element, and hence possess the ability to recruit initiation factors to the locus. Maps of the origin loci are provided in Figure 2 with replicator sites and other gene landmarks indicated. Though all three origins have regions documented to function as replicators, the precise site where DNA synthesis begins is not necessarily restricted to the replicator element. Both the c-myc and β-globin origin loci have been reported to initiate DNA synthesis from multiple potential sites throughout broad loci in human cells (Kamath and Leffak, 2001; Trivedi et al, 1998; Waltz et al, 1996), with any given site being used with a specific, nonrandom frequency. In contrast, initiation of DNA synthesis at the lamin B2 locus has been mapped at the nucleotide level (Abdurashidova et al, 2000), and thus more closely recapitulates the simpler replication origin model of budding yeast.

To investigate whether the level of histone acetylation at specific DNA sites correlates with the frequency of initiation of DNA replication at that site, quantitative, real-time PCR (Q-PCR) primers were designed to amplify several sites within each of the three origin loci (Figure 2, Table 1). Asynchronously

Figure 2. Maps of human replication origin loci.

The human c-myc, lamin B2, and β-globin loci are labeled with approximate locations of sequence-tagged sites (STSs) analyzed by quantitative real-time PCR (Q-PCR) (up-pointing arrows). Also provided are the distances between neighboring primer sets within each locus. Labeled on each locus are the approximate size of the chromosomal domain (double headed arrows) and the location of previously described replicator elements (dark gray bars). In the c-myc and β-globin maps the gene- or exon-encoding regions are indicated as light gray boxes. At the lamin B2 locus the hatched arrows represent the gene-encoding regions, and the major start site of bidirectional DNA synthesis is indicated by the oval and corresponding arrows.

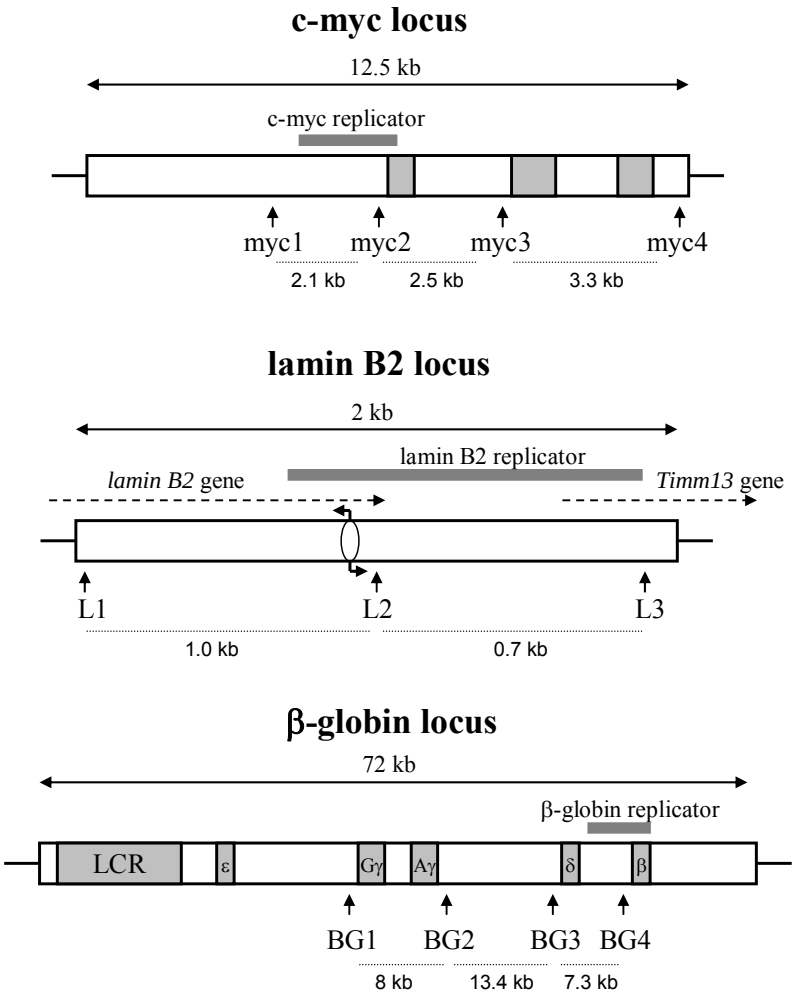

c-myc locus
12.5 kb
c-myc replicator
myc1
myc2
myc3
myc4
2.1 kb
2.5 kb
3.3 kb
lamin B2 locus
2 kb
lamin B2 replicator
lamin B2 gene
Timm13 gene
L1
L2
L3
1.0 kb
0.7 kb
β-globin locus
72 kb
β-globin replicator
LCR
ε
Gγ
Aγ
δ
β
BG1
BG2
BG3
BG4
8 kb
13.4 kb
7.3 kb

Table 1. Q-PCR primers used in this study.

The sequence of each primer used in Q-PCR analyses is provided with the corresponding location from the GenBank nucleotide database. Upper primers are denoted '-U' and lower primers '-L'. Coordinates of the primers refer to GenBank accession numbers U01317 (β-globin), M94363 (lamin B2), and HUMMYC (c-myc). The myc1 primer pair coordinates refer to GenBank accession number AF176208. The sequences of the random sequence primers used in additional PCRs are also provided.

Primer	Primer Sequence	Location
BG1-U	TTAACTTCCAAAGAACAAGTGCAA	33029-33054
BG1-L	CGGCTAAAAGACCAGAAAGATACAT	33082-33107
BG2-U	CACTTGTACACGGGAGTTCTGC	41168-41190
BG2-L	TCCTGTCCTATAGAGGTCTTTCTTCC	41224-41250
BG3-U	AAACCCTGCTTATCTTAAACCAACC	54653-54678
BG3-L	ACTCTGCCCTGCCTTTTATGC	54707-54728
BG4-U	CAGGAGCAGGGAGGGCA	62073-62090
BG4-L	AAGCAAATGTAAGCAATAGATGGCT	62122-62147
L1-U	TCTGTTTCCCACCTTCATAGCA	2908-2929
L1-L	ACCAAGACCCACCCCCAG	2978-2995
L2-U	ATGAAGCGGATGTCTAAGAAAG	4039-4063
L2-L	CGCCTGGGTCCTGTTTACAC	4105-4124
L3-U	AGAGGATGACGGACAAGTGTTTC	4821-4843
L3-L	CTCACCTGCTCGGAGTTGTCTA	4875-4896
50-01	GTGCAATGAG	GENOSYS GEN1-50 KIT
50-08	GGAAGACAAC	

growing HeLa cells were harvested for either ChIP analysis or for nascent strand abundance measurements. The ChIP protocol used in these studies employed formaldehyde to covalently crosslink histones and other proteins at their chromosomal DNA binding sites. Using antibodies recognizing tetra-acetylated histone H4 (AcH4), DNA sequences bound to tetra-acetylated histone H4 could be enriched. Finally, Q-PCR was employed to amplify specific DNA sequences in the immunoprecipitated DNA to determine the relative level of AcH4 at the PCR-amplified site. Similarly, the same primer sets were used to amplify short, 1- to 2-kb nascent DNA strands isolated from HeLa cells loaded and electrophoresed on an alkaline agarose gel.

Although limited in resolution due to the limited number of sites investigated, the highest level of acetylated histone H4 (AcH4) was found within or near the replicator elements at the early firing c-myc and lamin B2 replication origins (Figure 3A). Since these origins initiate synthesis early in S phase, it is possible that the more open chromatin structure that acetylated histone H4 provides may contribute to this early firing activity. In contrast, sites in the β-globin locus showed significantly less enrichment for AcH4, an expected characteristic of late-replicating chromosomal domains. Moreover, since the globin genes are not transcribed in HeLa cells, the chromatin may be less acetylated to prevent activation of transcription.

Interestingly, nascent strand abundance assays showed that the sites within the c-myc and lamin B2 loci (STS–myc2 and –L2, respectively) that showed the greatest enrichment for AcH4 were also the sites used with the greatest frequency for replication initiation (Figure 3B) and correspond to the documented replicator elements. Sites more distal to the replicator elements showed both reduced initiation site usage and lower AcH4 levels. There appeared to be no correlation between histone acetylation and initiation site selection at the β-globin locus, although this may be due to the large size of this domain (~72 kb) and the fact that only a few STS were examined. Additional experiments would be required to draw a clear conclusion. A similar lack of an obvious correlation between initiation site usage and histone acetylation was also seen at the β-globin locus in chicken cells (Prioleau et al, 2003).

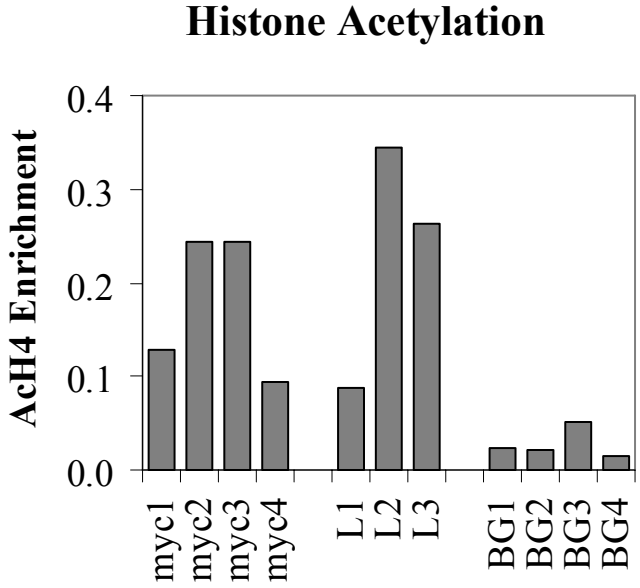
The histone deacetylase inhibitor TSA increases global histone acetylation levels

Since there appeared to be at least a rough correlation between the level of histone acetylation and the tendency of a site to initiate DNA replication at two replication origins, it was of interest whether modifying the acetylation state of histones within the replication origin loci would alter the selection of

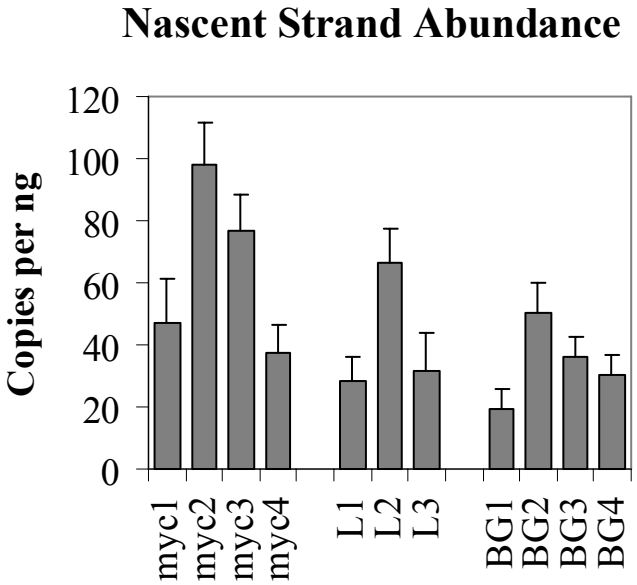
Figure 3. Relative levels of histone acetylation and replication origin activity at human replication origin loci.

(A) The level of histone acetylation was determined by chromatin immunoprecipitation with an anti-acetylated-histone H4 antibody (AcH4) and Q-PCR. The data are calculated as the difference in amount of DNA precipitated by anti-AcH4 antibody and normal rabbit serum as a fraction of the total input cross-linked DNA [(AcH4-NRS)/Input]. The data presented are average values from triplicate Q-PCRs, and the standard deviation was less than 5% of the mean. These data were provided by Maloy Ghosh. A simpler ratio of the amount of DNA precipitated by anti-H4 antibody to that precipitated with normal rabbit serum (AcH4/NRS) gave similar results. (B) Replication origin activity was determined by a Q-PCR based nascent strand abundance assay. The data are presented as an average (and standard deviation) from three independent preparations of nascent DNA analyzed by Q-PCR in triplicate. The total amount of nascent DNA in each preparation was determined by OliGreen fluorescence quantification.

A


Histone Acetylation
AcH4 Enrichment
0.4
0.3
0.2
0.1
0.0
myc1
myc2
myc3
myc4
L1
L2
L3
BG1
BG2
BG3
BG4

B


Nascent Strand Abundance
Copies per ng
120
100
80
60
40
20
0
myc1
myc2
myc3
myc4
L1
L2
L3
BG1
BG2
BG3
BG4

initiation sites. To test this hypothesis, the histone deacetylase inhibitor trichostatin A (TSA) was employed to increase the level of histone acetylation both at these three replication origin loci and throughout the genome. TSA has been reported to activate the transcription of a many genes (Gray et al, 2004), presumably because of its ability to inhibit some of the enzymes (HDACs) that contribute to restricting gene expression.

To confirm that TSA increases the levels of acetylation on histones in HeLa cells, western blots were performed using whole cell extracts from HeLa cells treated with TSA for 4 to 24 hours (Figure 4A). TSA rapidly and significantly increased the level of AcH4 within 4 hours. The effect of TSA on AcH4 level was transient and reversible though, since the AcH4 level begins to decrease by 12 hours as the drug was degraded, and returned to background levels by 18 hours after initial treatment. These data argue that TSA can be used to induce a rapid, transient change in the level of bulk histone acetylation in human cells.

Increased histone acetylation might be expected have both local effects on the interaction of nucleosomes with DNA and on the folding of higher order chromatin structures. To determine whether TSA alters the level of chromatin compaction, HeLa cells treated with TSA were stained with the intercalating DNA dye acridine orange after denaturation of the chromosomal DNA under acidic conditions. Acridine orange (AO) has metachromatic properties, such that under certain conditions it can differentially stain double-stranded versus single-stranded DNA (Darzynkiewicz and Carter, 1989; Darzynkiewicz, 1990). When intercalated into dsDNA and excited by blue light, AO emits green fluorescence. In contrast, when AO complexes with ssDNA it precipitates on the DNA and is characterized by red luminescence. AO has routinely been used to accurately determine the percentage of cells that are undergoing mitosis (Darzynkiewicz et al, 1977b), due to the observation that DNA in compact, mitotic chromatin is more susceptible to acid-induced denaturation. Thus in fixed, acid-denatured mitotic cells stained with acridine orange, more of the dye binds to the ssDNA that is generated, and hence a greater percentage of the dye fluoresces red. Similar observations have been made in cells treated with compounds that modify chromatin structure, such as the HDAC inhibitor sodium butyrate, with a more compact chromatin structure being correlated with increased red fluorescence of AO (Darzynkiewicz 1990). HeLa cells treated with TSA for 4 hours were harvested, fixed, and their chromosomal DNA denatured by brief acid treatment. After neutralization, the DNA was stained with acridine orange and the cells analyzed by

flow cytometry. As indicated in Figure 4B, a decrease in red fluorescence was observed after treatment with TSA, consistent with a more open chromatin structure and chromosomal DNA less susceptible to acid-induced denaturation. A corresponding increase in green fluorescence was also observed, indicating the dye was bound to DNA in one of two possible states.

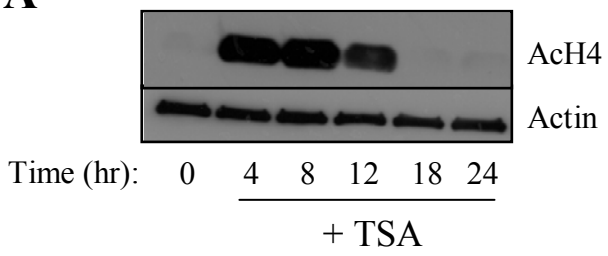
Since TSA is known to alter the expression of many genes in HeLa cells, and the effects of some of these change in gene expression are manifested in morphological alterations to the cells, we investigated using phase contrast microscopy the morphology of HeLa cells after treatment with TSA. As previously indicated (Li and Wu, 2004), TSA caused HeLa cells to become more elongated with filamentous protrusions (Figure 4C), possibly due to the progressive rearrangement and dispersion of the cytoskeleton. Expression of the actin binding protein gelsolin has been reported to be significantly increased in HeLa cells treated with TSA and play a role in the morphological changes to the cells (Hoshikawa et al, 1994). These morphological changes were transient though, as cells returned to their normal oval or polygonal morphology within 24 to 30 hours after initial treatment with TSA (data not shown), slightly delayed relative to the time at which the AcH4 levels returns to background levels (Figure 4A). Importantly, no indication of cell death induction was observed after treatment with TSA under these conditions. Together these data indicate that the low dose of TSA employed is sufficient to cause an increase in the acetylation level of histone H4 and to alter the level of chromatin compaction in HeLa cells. Moreover, the morphological changes induced by TSA are consistent with previous observations and indicates that TSA can exert its effects at specific chromosomal (promoter) sites.

Clinically HDAC inhibitors such as TSA are of interest because of their ability to inhibit cancer cell proliferation (Hoshikawa et al, 1994; Liu et al, 2006). To determine whether the low-level dose of TSA employed causes changes in HeLa cell proliferation or cell cycle progression, cells treated with TSA for 4 to 24 hours were analyzed by flow cytometry after staining with propidium iodide. The flow cytometry profiles indicate that TSA induced arrests of the cell cycle at both G1 and G2 phases (Figure 5A), as previously reported (Kim et al, 2000; Li and Wu, 2004). By 12 hours after TSA treatment, nearly all the cells were in either G1 or G2 phases, based on the DNA content of the cell population, indicating both G1 and G2 phase checkpoint activation. Consistent with data suggesting that the effect of TSA on AcH4 level was transient (Figure 4A), by 18 hours after TSA treatment cells arrested in G2 began to move through

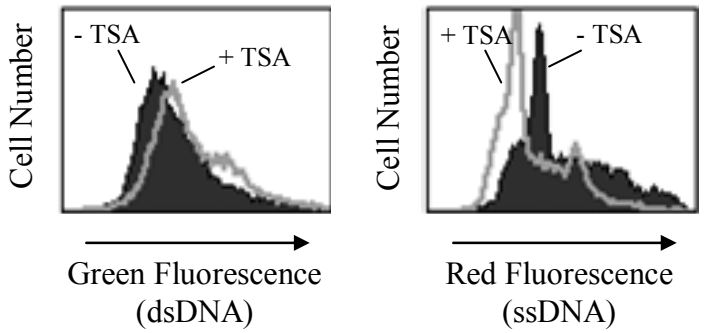
Figure 4. The histone deacetylase inhibitor trichostatin (TSA) causes a transient increase in histone acetylation, alters chromatin compaction, and changes cell morphology.

(A) HeLa cells were grown for indicated number of hours in the presence of 100 ng/ml TSA before preparing whole cell extracts and analyzing by SDS-PAGE and western blotting with anti-acetylated histone H4 and anti-actin antibodies. (B) HeLa cells treated for 4 hours with 100 ng/ml TSA were analyzed in an acridine orange-based flow cytometry assay to detect the level of chromatin compaction. Red fluorescence indicates precipitation of the dye on ssDNA, an event that occurs in cells that have DNA easily denatured under acidic conditions. A decrease in red fluorescence indicates a less compact chromatin structure, and hence a DNA duplex less susceptible to acid-induced denaturation. Green fluorescence indicates the intercalation of the acridine orange dye into dsDNA. (C) Phase contract microscopy of untreated HeLa cells (left) or cells treated for 4 hours with 100 ng/ml TSA (right). The effect of TSA on cell morphology is reversible, as cells return to a normal morphological state within 24-30 hours after initial treatment (not shown).

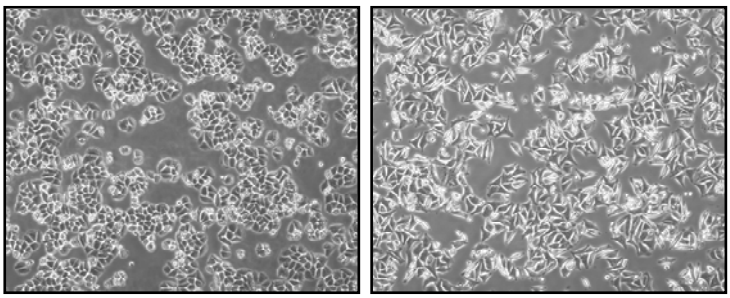
A


AcH4
Actin
Time (hr): 0 4 8 12 18 24
+ TSA

B


Cell Number
- TSA
+ TSA
Green Fluorescence
(dsDNA)
Cell Number
+ TSA
- TSA
Red Fluorescence
(ssDNA)

C


- TSA
+ TSA

mitosis and enter a new G1 phase. Moreover, by 24 hours after TSA treatment approximately half the cell population had begun to enter a new S phase, as indicated both by DNA content analysis (Figure 5A) and by determining the percentage of cells incorporating BrdU (Figure 5B), a thymidine analog commonly used to detect cells synthesizing DNA. Higher does of TSA (>300 ng/ml) maintained the cell cycle blocks longer (Figure 5B and data not shown). Thus these results indicate that although TSA induced cell cycle arrests, these blocks were transient, and cells treated with a low dose of TSA for a short period of time (4-8 hours) may be useful for investigating how TSA affects events occurring during S phase of the cell cycle.

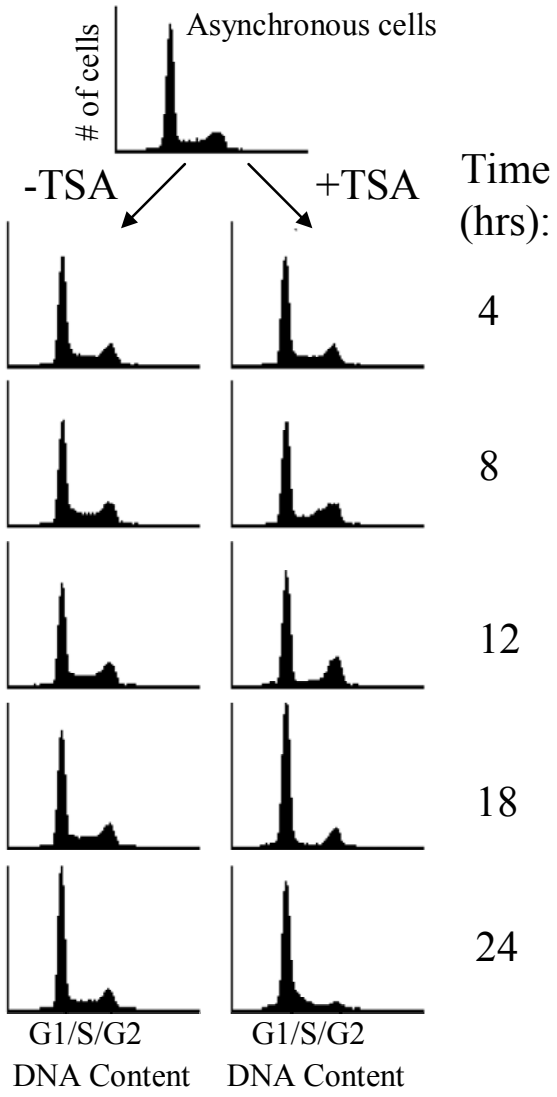
TSA promotes DNA synthesis at the G1/S transition

In an attempt to look at the effects of TSA specifically in S phase cells, HeLa cells were synchronized with either mimosine or aphidicolin. Mimosine is a plant amino acid useful for arresting cells in very late G1 phase, before the establishment of replication forks (Krude, 1999; Lalande, 1990). Aphidicolin inhibits the replicative DNA polymerases, and hence cells arrested with this drug become blocked at a very early stage in S phase. By removing drug-containing medium and replacing with drug-free medium, either mimosine- or aphidicolin-arrested cells being to synthesize DNA and move through S phase. To investigate whether TSA can affect the accumulation of replicated DNA, HeLa cells were released from either a mimosine- or aphidicolin-induced cell cycle arrest into fresh medium containing TSA. As shown in Figure 6, TSA did not prevent these arrested cells from synthesizing DNA, though not all the cells released from a mimosine block into TSA entered S phase. Interestingly, there appeared to be very little difference in the rate at which cells replicated their DNA after release from aphidicolin into medium lacking or containing TSA, as previously reported (Nair et al, 2001). TSA-treated cells became arrested at G2 phase though, as previously indicated (Figure 5). In contrast, cells released from mimosine into fresh medium containing TSA completed S phase more rapidly, since all of the TSA-treated cells that moved into S phase were observed to have a DNA content of G2 cells by 12 hours, whereas a significant percentage of the non-TSA-treated cells has synthesized less DNA by this time point (see Appendix D). The observed difference between the mimosine and aphidicolin block and release experiments indicated that TSA exerted an effect at some point between where these two compounds blocked the cell cycle. Since

Figure 5. Low dose TSA treatment induces reversible cell cycle blocks.

(A) Asynchronously growing HeLa cells were left untreated or treated with 100 ng/ml TSA for the indicated period of time, fixed and stained with propidium iodide, then analyzed for DNA content by flow cytometry. Cells treated with TSA at 300 ng/ml retain the G2 arrest for at least 30 hours (data not shown). (B) Cells were treated with indicated concentrations of TSA for 24 hours, but pulsed during the last half hour with BrdU. Cells were then analyzed by immunofluorescence flow cytometry to determine the number of cells in S phase. Data are the average and range of two independent experiments.

A


Asynchronous cells
# of cells
-TSA
+TSA
Time (hrs):
4
8
12
18
24
G1/S/G2 DNA Content
G1/S/G2 DNA Content

B

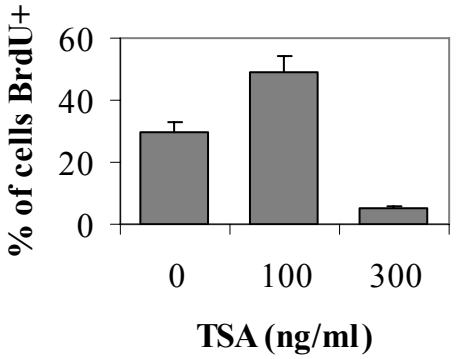
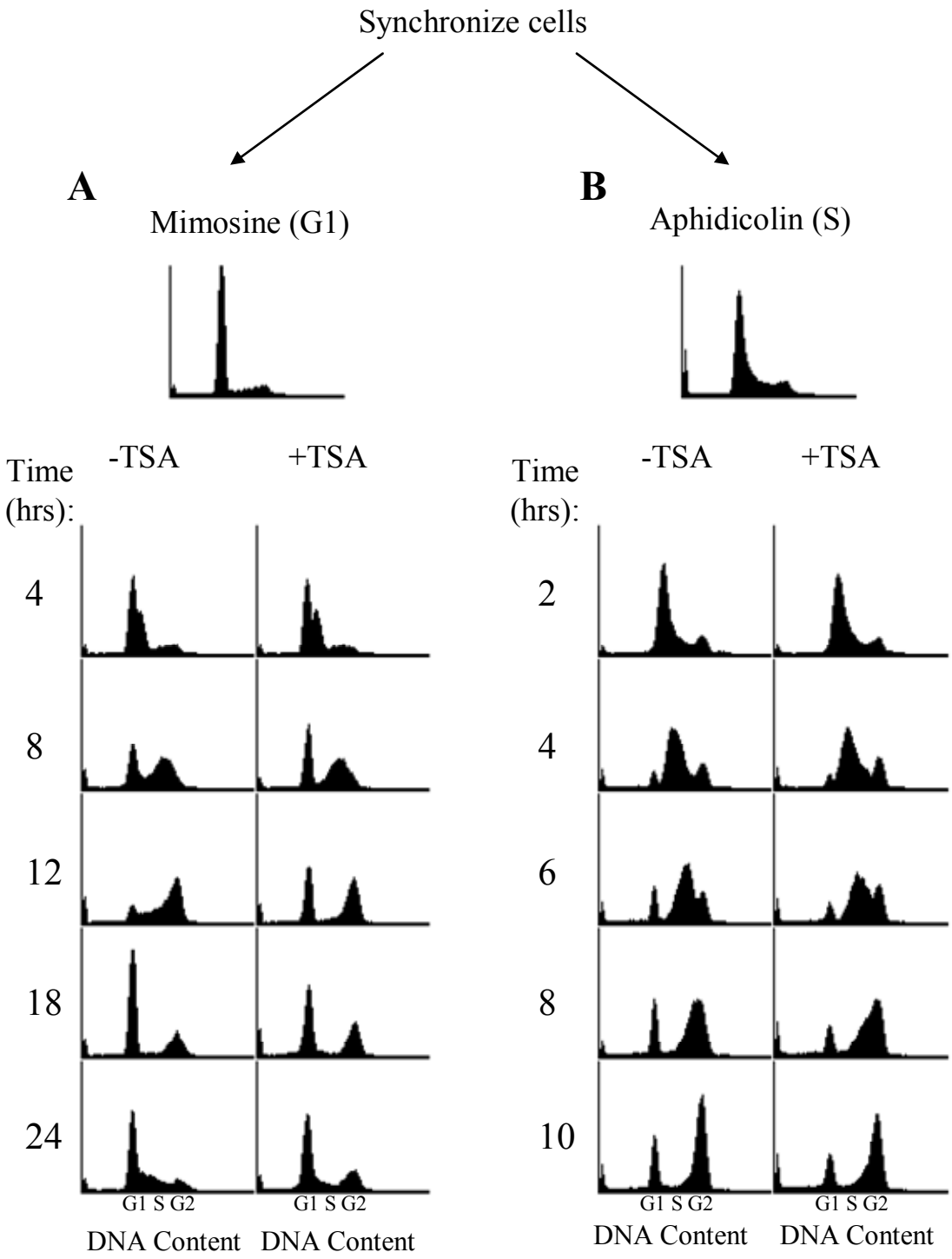

% of cells BrdU+
60
40
20
0
0
100
300
TSA (ng/ml)

Figure 6. TSA promotes DNA replication at an early stage of S phase.

Cells were synchronized for either 18 hours with 200 μM mimosine (A) or for 24 hours with 1 μg/ml aphidicolin (B). The medium was then replaced with fresh medium lacking or containing 100 ng/ml TSA. At the indicated times cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. A quantification of the cell cycle distribution for (A) is provided in Appendix D.


Synchronize cells
A
Mimosine (G1)
B
Aphidicolin (S)
Time (hrs):
-TSA
+TSA
4
8
12
18
24
Time (hrs):
-TSA
+TSA
2
4
6
8
10
G1 S G2
DNA Content
G1 S G2
DNA Content
G1 S G2
DNA Content
G1 S G2
DNA Content

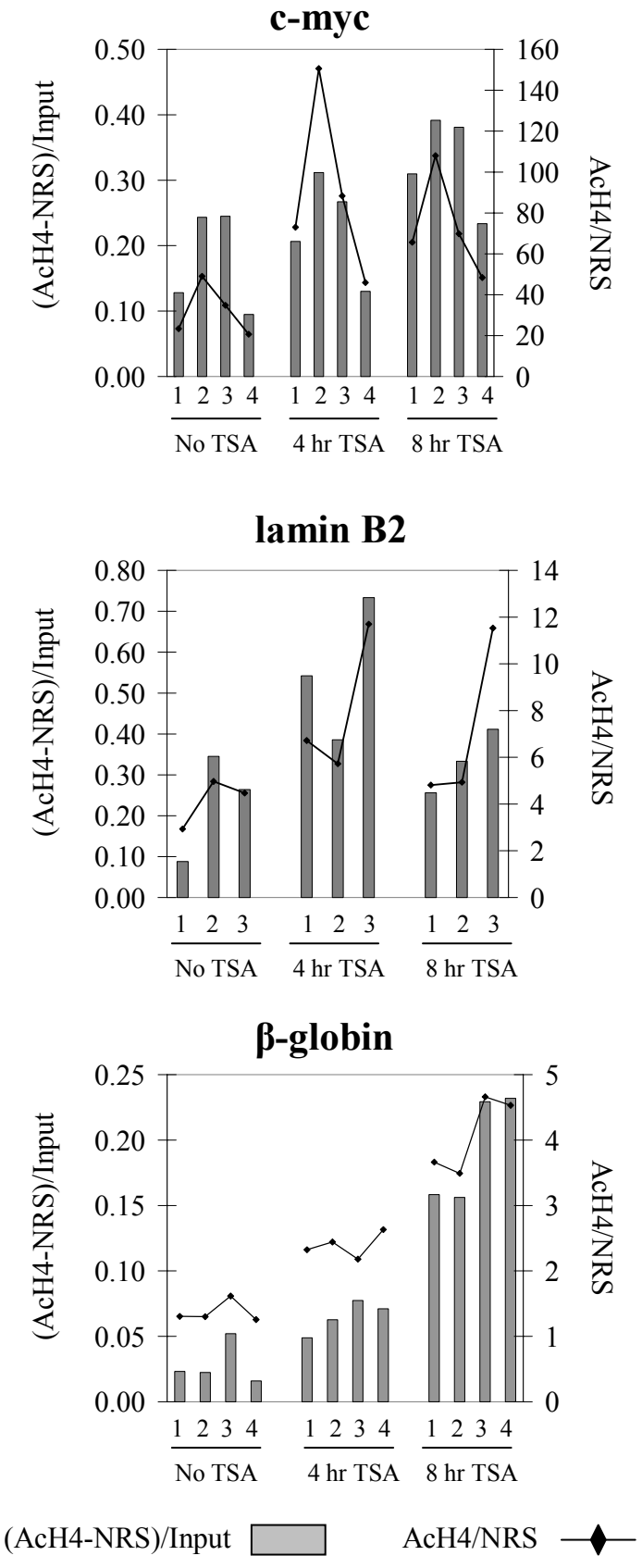
the major reported difference is in the absence or presence of established replication forks between a mimosine- and aphidicolin-block, respectively, these data argue that TSA promoted the establishment of replication forks at origins of replication. Since in aphidicolin-arrested cells many replication origins have already fired and the replication fork was simply stalled due to polymerase inhibition, it was likely that the ability of TSA to promote S phase progression in mimosine-released cells was through an ability to specifically act at replication origins.

TSA increases histone acetylation levels at replication initiation sites

Since TSA increased the acetylation state of histone H4 in bulk chromatin (Figure 4A) we were interested in whether these effects were apparent at sites within or near replication origin loci. ChIP was used to enrich for sequences bound by tetra-acetylated histone H4, and these sites were probed using specific primer sets by Q-PCR as indicated in Figure 2. At the c-myc, lamin B2, and β-globin replication origins TSA induced an increase in AcH4 state at all sites investigated within 4 hours (Figure 7), although some sites were affected to a greater extent than other sites. For example, STS-L2 in the lamin B2 locus showed little change in AcH4 level after treatment with TSA, whereas sites flanking STS-L2 (STS-L1 and –L3) showed a much more robust increase in AcH4 levels. These results indicate that some sites may be differentially affected by TSA inhibition, presumably due to the balance of HAT and HDAC activity at any given site, and similar results were observed at the c-myc locus. At the β-globin locus the initial AcH4 pattern among the four sites investigated was abolished after TSA treatment, with all sites showing large (4- to 12-fold) increases in AcH4 level after 8 hours of treatment. The observation that sites in the β-globin locus are affected to a greater extent by TSA treatment than sites in either the c-myc or lamin B2 origins was consistent with the β-globin locus being in normally a repressed chromatin structure. This repressive structure, presumably partially the result of HDACs acting at the locus to keep the chromatin in a closed conformation, is expected of chromosomal regions that are not transcribed and that are replicated late in S phase. In summary, TSA appeared to change the pattern of histone acetylation at replication origin loci, abolishing the normal pattern found in untreated, asynchronously growing HeLa cells. The change in pattern may serve to remove any preference for specific regions of DNA being in an open, accessible conformation for protein binding.

Figure 7. TSA causes hyperacetylation of histones at replication origin loci in human cells.

ChIP was used to enrich for DNA sequences bound to chromatin with acetylated histone-H4 at three replication origin loci from either untreated cells or cells treated with 100 ng/ml TSA for 4 or 8 hours. The STSs analyzed are abbreviated by numbers for each locus. DNA was quantitated by Q-PCR and is presented as both (AcH4-NRS)/Input and AcH4/NRS. The data are average Q-PCR values from samples run in triplicate, and were provided by Dr. Maloy Ghosh. The standard deviation (not shown) was under 5% of the mean for each sample.


c-myc
(AcH4-NRS)/Input
AcH4/NRS
0.50
0.40
0.30
0.20
0.10
0.00
160
140
120
100
80
60
40
20
0
1 2 3 4 1 2 3 4 1 2 3 4
No TSA
4 hr TSA
8 hr TSA
lamin B2
(AcH4-NRS)/Input
AcH4/NRS
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
14
12
10
8
6
4
2
0
1 2 3 1 2 3 1 2 3
No TSA
4 hr TSA
8 hr TSA
β-globin
(AcH4-NRS)/Input
AcH4/NRS
0.25
0.20
0.15
0.10
0.05
0.00
5
4
3
2
1
0
1 2 3 4 1 2 3 4 1 2 3 4
No TSA
4 hr TSA
8 hr TSA
(AcH4-NRS)/Input
AcH4/NRS

TSA increases MCM levels on chromatin

Because TSA increased acetylation levels at replication origin loci in human cells, it was of interest to determine whether the binding of replication proteins to these replication origins was altered after TSA treatment. Moreover, since TSA exerted its effects on histone acetylation globally throughout the genome, and can promote S phase entry, we were interested in whether TSA affected the binding of replication proteins to bulk chromatin. HeLa cells were treated with TSA for 4, 8, or 12 hours and then harvested for the isolation of chromatin-bound proteins. As shown in Figure 8A, TSA did not affect the total cellular expression of any replication proteins investigated. Interestingly though, when examining the level of replication proteins on chromatin, we noticed that two members of the MCM helicase complex were elevated on chromatin after TSA treatment. Although Mcm3 and Mcm7 were elevated on chromatin, other replication proteins such as Orc2 and PCNA did not appear to be significantly affected by TSA treatment (Figure 8B). Recently it was shown that Cdt1, a component of the machinery that loads MCMs at replication origins, interacts both in *Xenopus* egg extracts and in human cells with the histone acetyltransferase HBO1 (Iizuka et al, 2006), where it functions to load additional MCMs at replication origins. Our results are consistent with this data, such that inhibition of HDAC activity promoted MCM loading on chromatin. A similar observation was made when Orc2 and Mcm3 levels at the c-myc origin were studied using ChIP (Ghosh et al, 2006). Although TSA decreased the total crosslinking of both of these proteins at the c-myc locus, the pattern of Orc2 distribution was unaltered whereas MCMs showed a broadened distribution along the origin locus (Ghosh et al, 2006).

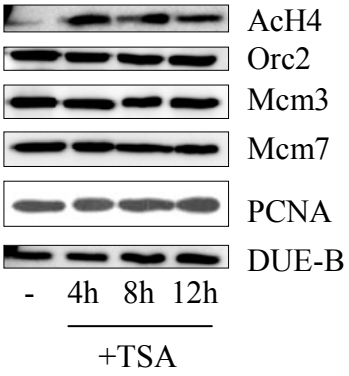
Replication origin activity is altered by TSA treatment

The loading of multiple MCM complexes at replication origins has been hypothesized to be responsible for the observation that in somatic human cells, replication initiates from any of a number of potential sites within a given locus (Alexandrow et al, 2002; Dijkwel and Hamlin, 2002; Gilbert, 2001). Our data showing that TSA altered histone acetylation at origins and increased the level of chromatin-bound MCMs suggested that the pattern of replication origin selection may be altered after TSA treatment. To investigate this hypothesis, short 1- to 2-kb nascent DNA strands were isolated from HeLa cells treated with TSA for 4, 8, or 24 hours. The purified nascent DNA was then analyzed using Q-PCR to determine whether the frequency of initiation site usage was altered after treatment with TSA. The most striking

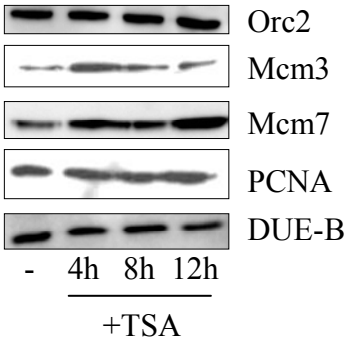
Figure 8. TSA causes an increase in chromatin-bound MCMs.

Untreated HeLa cells or cells treated with 100 ng/ml TSA for 4, 8 or 12 hours were harvested to make whole cell extracts (A) or instead lysed to release nuclei for formaldehyde cross-linking (B). Cross-linked chromatin was purified as described in the methods section. After reversing the cross-links by boiling and then sonication in SDS-PAGE sample buffer, chromatin-bound proteins were analyzed by SDS-PAGE and western blotting with antibodies against the indicated proteins. Lysate from an equivalent number of cells was loaded in each lane.

A


WCE
AcH4
Orc2
Mcm3
Mcm7
PCNA
DUE-B
- 4h 8h 12h
+TSA

B


Chromatin
Orc2
Mcm3
Mcm7
PCNA
DUE-B
- 4h 8h 12h
+TSA

observation was that TSA treatment caused an overall decrease in initiation site usage at most of the sites investigated within the c-myc, lamin B2, and β-globin replication origin loci (Figure 9). That total Orc2 levels on chromatin remain unchanged after TSA treatment (Figure 8B) but are decreased at the c-myc origin (Ghosh et al, 2006), suggest that ORC is being redistributed to new origin sites (discussed below). Furthermore, OliGreen quantitation of total nascent DNA indicated an equivalent amount of nascent DNA was isolated from untreated and TSA-treated cells (data not shown), indicating that there were an equivalent number of replication origins firing in cells treated or not with TSA, and that new or other uncharacterized sites are used with a greater frequency after TSA treatment.

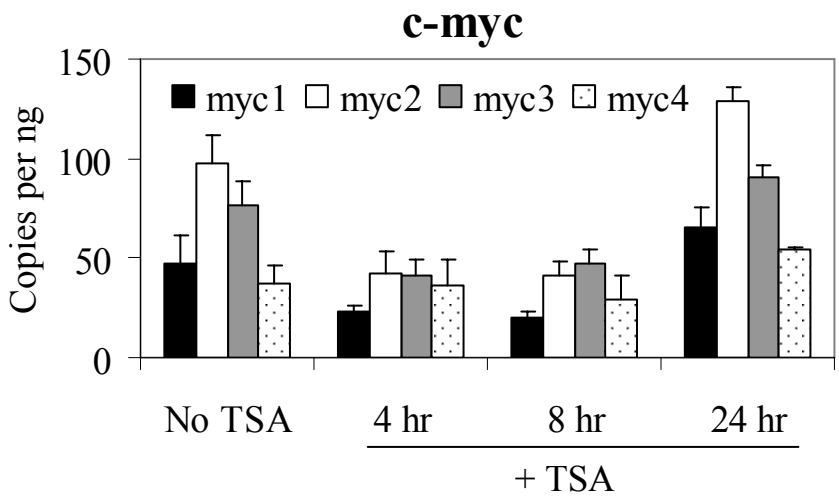
Within the individual replication origin loci though, TSA appeared to abolish the previous pattern of initiation site selection, such that sites previously used with highest frequency are no longer preferred after TSA treatment. At the c-myc locus, STS-myc2 showed the highest abundance of nascent strands, suggesting that it was a preferred site for replication initiation within the c-myc replication origin locus. After TSA treatment for 4 or 8 hours, though, STS-myc2 no longer showed preferred initiation site usage, with sites STS-myc2, -myc3 and –myc4 being used at approximately the same frequency. Interestingly, this loss of loss of initiation site preference was correlated with the alteration in histone acetylation along the locus.

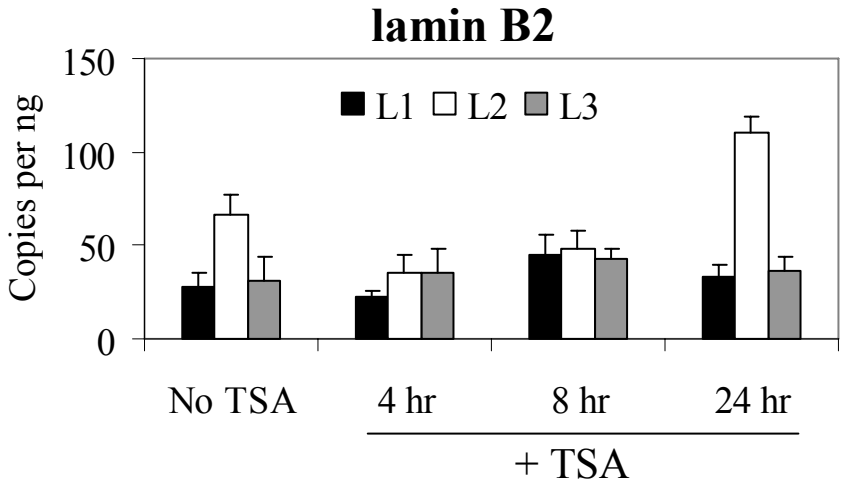
At the lamin B2 locus, which typically shows very restricted initiation site selection, TSA abolished STS-L2 as the preferred site for initiation, with STS-L1 and –L3 displaying similar levels of nascent strands after treatment with TSA. The normally restricted region over which replication typically initiates was broadened after treatment with TSA. The pattern returned to that in untreated cells 24 hours after TSA treatment though, presumably due to global histone acetylation levels returning to that found in untreated cells at that time (Figure 4A). The overall increase in nascent strands at the lamin B2 and c-myc origins at this time point was likely due to the increased number of cells in early S phase (Figure 5).

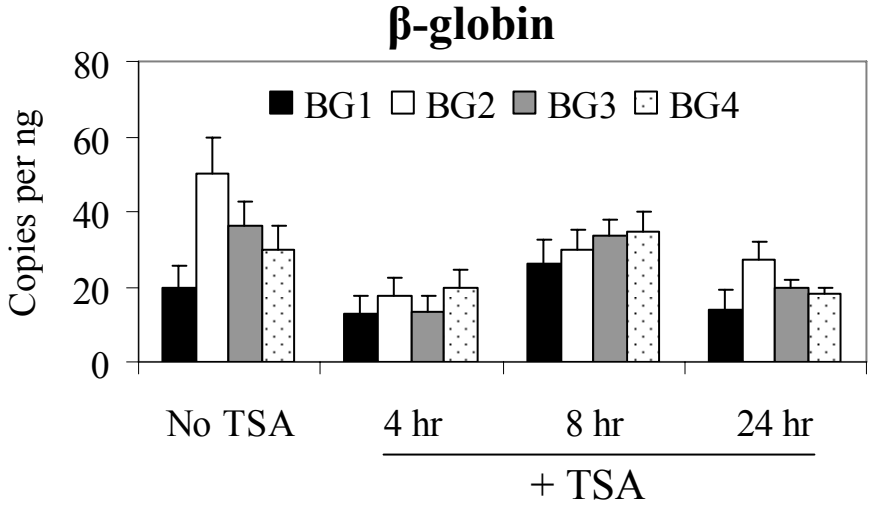
At the late firing β-globin locus TSA also induced a loss of initiation site preference, with each site being used with approximately equal frequency 8 hours after TSA treatment. Similar to that found at the c-myc and lamin B2 origins, the pattern of origin activity returned to normal 24 hours after TSA

Figure 9. TSA alters the pattern of replication origin activity in human cells.

Asynchronously growing HeLa cells were treated for 4, 8, or 24 hours with 100 ng/ml TSA and loaded on alkaline gels for isolation of 1- to 2-kb nascent DNA. DNA was then purified, quantitated by OliGreen fluorescence, and used in Q-PCR analyses to measure the abundance of newly synthesized DNA at specific STSs. The data are presented as the average (with standard deviation) of triplicate Q-PCR analysis on at least three independent preparations of nascent DNA.


c-myc
150
100
50
0
Copies per ng
myc1
myc2
myc3
myc4
No TSA
4 hr
8 hr
24 hr
+ TSA


lamin B2
150
100
50
0
Copies per ng
L1
L2
L3
No TSA
4 hr
8 hr
24 hr
+ TSA


β-globin
80
60
40
20
0
Copies per ng
BG1
BG2
BG3
BG4
No TSA
4 hr
8 hr
24 hr
+ TSA

treatment. Because of the enrichment for early S phase cells at this time point, the total level of nascent strands from the β-globin locus was decreased compared to untreated cells.

To confirm that it was newly synthesized DNA that was affected by TSA treatment, and not simply an artifact of the technique used to isolate nascent DNA, HeLa cells were pulsed with BrdU after treatment with TSA. Nascent DNA was isolated by alkaline gel electrophoresis, purified, and finally immunoprecipitated with anti-BrdU antibody. This precipitated DNA was then used in Q-PCRs to examine the abundance of c-myc, lamin B2, and β-globin sequences in the input and immunoprecipitated, BrdU-labeled DNA. As shown in Figure 10, TSA decreased the abundance of nascent strands at the primary initiation sites in the c-myc, lamin B2, and β-globin replication origin loci. The observation that the abundance values in the precipitated material were >80% of that found in the input, non-immunoprecipitated DNA sample confirmed that the observed changes in abundances from DNA isolated from alkaline gels was due to changes in newly synthesized DNA.

Together these data suggest that TSA affected the usage of specific sites for the initiation of DNA replication, probably through an alteration in histone acetylation that abolished the chromatin structure that usually gave rise to characteristic, preferred initiation sites.

TSA promotes initiation at new chromosomal sites

Since we observed absolute decreases in nascent strand abundance per nanogram of newly synthesized DNA at most sites after 4 hours of TSA treatment, there must be other, normally inefficient origin sites used with greater frequency after treatment with TSA. To test this hypothesis, random sequence 10-mer primers (Table 1) were used to amplify unknown genomic sites from untreated or TSA treated cells. These primers generated products of ~300 to 1400 bp in size that were visualized as specific bands on agarose gels stained with ethidium bromide (Figure 11). An equal amount of total template nascent DNA was used in each PCR from the different time points, so any observed difference in band intensity was due to a difference in origin activity from the uncharacterized genomic site. The changes in patterns observed using different primers implied that some sites increased and others decreased in frequency as initiation sites for DNA replication after treatment with TSA. Importantly, the patterns returned to normal by 48 hours after TSA treatment, further arguing that the low dose of TSA employed did not have any long-

Figure 10. TSA decreases the abundance of BrdU-labeled nascent strands at primary initiation sites. Untreated HeLa cells or cells treated for 4 hours with 100 ng/ml TSA were incubated for 30 minutes with BrdU before isolation of 1- to 2-kb nascent DNA by alkaline agarose gel electrophoresis. The abundance of STS-myc2, -L2, and –BG2 were analyzed by Q-PCR as the amount present in the purified DNA (input) or the amount of DNA immunoprecipitated with anti-BrdU antibody. The data are presented as the average and standard deviation from triplicate Q-PCR analysis from a single preparation of BrdU-labeled nascent DNA.

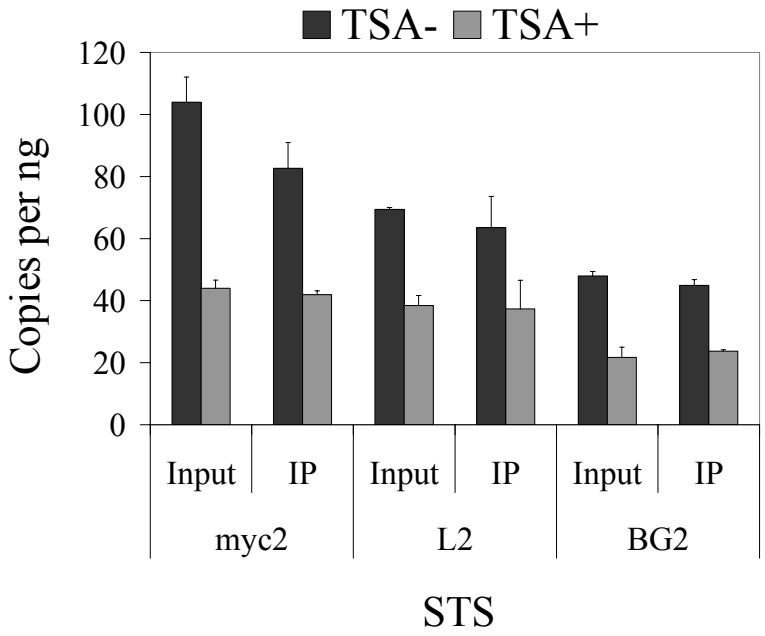
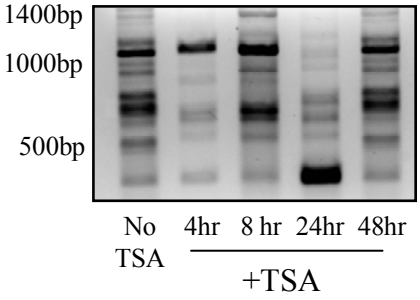

TSA-
TSA+
120
100
80
60
40
20
0
Copies per ng
Input
IP
Input
IP
Input
IP
myc2
L2
BG2
STS

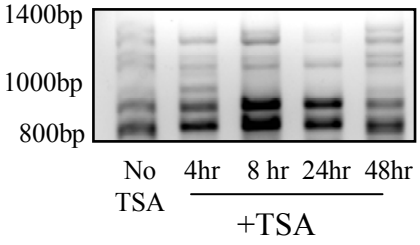
Figure 11. TSA alters initiation at uncharacterized sites.

Nascent DNA from cells treated with 100 ng/ml TSA for the indicated lengths of time were amplified by PCR using random 10-mer sequence primers 50-01 (A), 50-08 (B) or both 50-01 and 50-08 (C) and equal amounts of starting template nascent DNA. Products were purified, electrophoresed in 2% agarose gels and stained with ethidium bromide (inverse image shown).

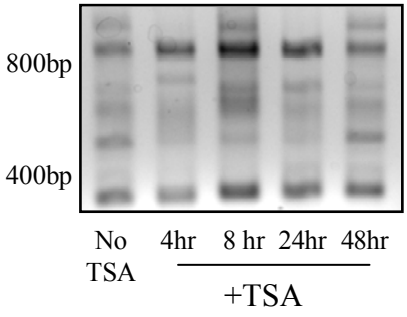
A


1400bp
1000bp
500bp
No TSA
4hr
8 hr
24hr
48hr
+TSA

B


1400bp
1000bp
800bp
No TSA
4hr
8 hr
24hr
48hr
+TSA

C


800bp
400bp
No TSA
4hr
8 hr
24hr
48hr
+TSA

lasting, irreversible effects on chromosome structure or regulation.

TSA causes origin firing earlier in S phase

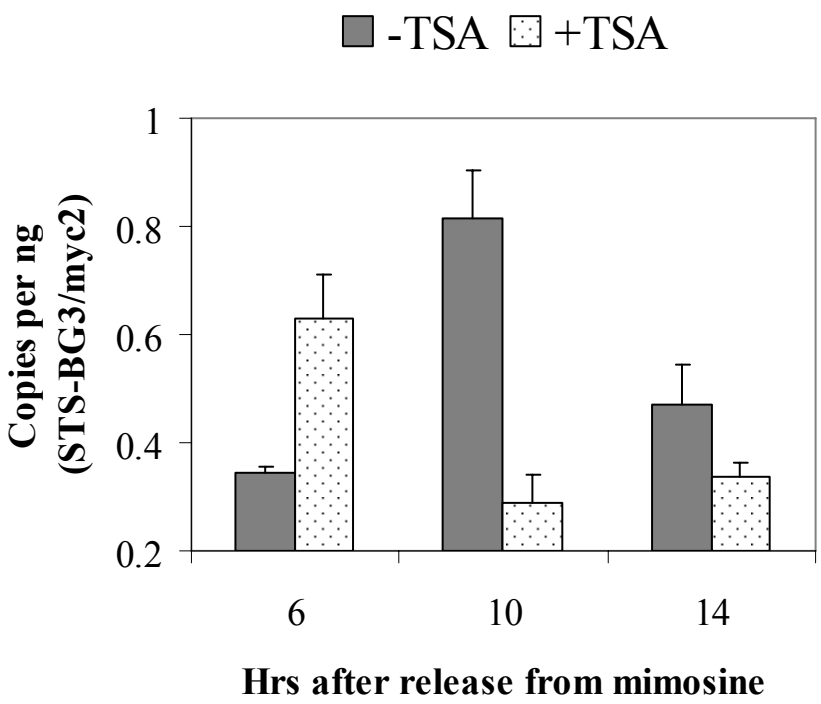
During our initial investigation into the effects of TSA on replication origin activity it was reported that in budding yeast the specific recruitment of a histone acetyltransferase to late-firing replication origins caused the origin to initiate DNA synthesis earlier in S phase (Vogelauer et al, 2003). To examine whether a similar phenomenon existed in human cells, HeLa cells were blocked in late G1 phase by treatment with mimosine, then released into fresh medium containing TSA. At specific time points after release, cells were harvested and nascent DNA prepared for use in Q-PCR. In untreated cells replication initiates from the β-globin locus (STS-BG3) primarily 10 hours after release from mimosine (Figure 12), consistent with the observation that the β-globin locus is replicated late in S phase. In contrast, after treatment with TSA, maximal nascent DNA from this site was observed only 6 hours after release from mimosine, suggesting that an effect of TSA was to allow initiation from late-firing origins earlier in S phase. The absolute decease in nascent strands coming from this site was consistent with data in Figure 9 that showed reduced origin activity at many previously characterized initiation sites. These results also suggested that one function of HDACs may be to restrict the time during S phase at which origins can be activated for the initiation of DNA synthesis.

Summary

The presented data suggest a role for histone acetylation in the regulation of DNA replication in human cells. Since a correlation appeared to exist between the level of histone acetylation and replication initiation at the early replication lamin B2 and c-myc loci, we used the histone deacetylase inhibitor TSA to increase the level of histone acetylation both at replication origin loci and throughout the genome. The results showed that this increased acetylation correlated with a loss of initiation site specificity, as well as initiation from new sites and initiations earlier in S phase. The increased origin usage could be a result of an increase in the number of MCMs on chromatin, since this increased MCMs on chromatin is thought to be responsible for the broadened zones of replication initiation in mammalian cells. Further work will need to be performed to understand the molecular mechanisms behind this regulation and to identify specific histone acetyltransferases and histone deacetylases that are involved in this process.

Figure 12. Cells treated with TSA initiate DNA synthesis from the late-firing β-globin origin earlier in S phase.

Mimosine-synchronized cells were released into fresh medium lacking or containing 100 ng/ml TSA for the indicated number of hours before isolation of 1- to 2-kb nascent DNA by alkaline agarose gel electrophoresis. The nascent DNA was analyzed by Q-PCR in triplicate reactions to determine the abundance at STS-BG3 and STS-myc2. The data are from two independent preparations of nascent DNA and are normalized to STS-myc2.


-TSA
+TSA
1
0.8
0.6
0.4
0.2
Copies per ng
(STS-BG3/myc2)
6
10
14
Hrs after release from mimosine

II. Identification of the novel replication origin binding protein DUE-B

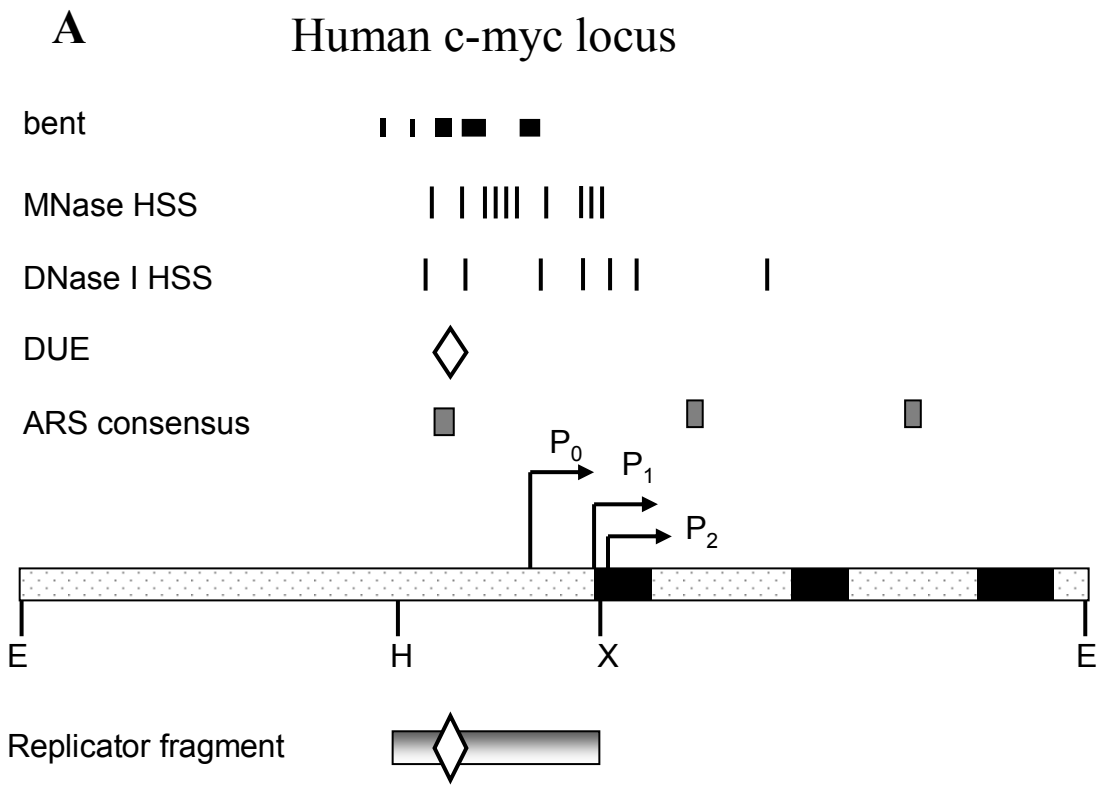
A DUE is an essential functional element of the c-myc replicator

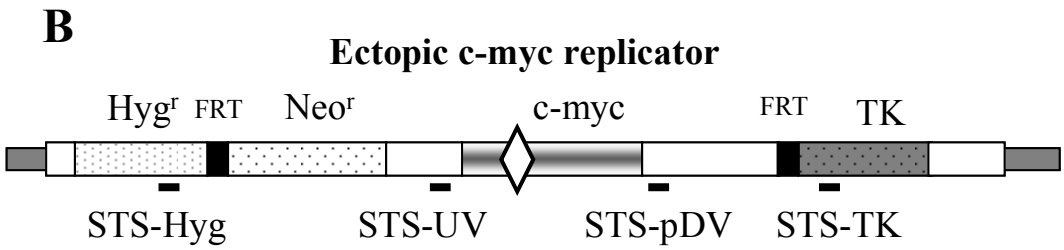
A 2.4-kb segment of DNA upstream of the human c-myc gene (Figure 13A) was demonstrated by our laboratory to function as a genetic replicator, based on its ability to induce the initiation of DNA replication when inserted at an ectopic chromosomal location (Liu et al, 2003; Malott and Leffak, 1999). The c-myc replicator fragment contains several nuclease hypersensitive sites and fixed nucleosomes that are maintained when the fragment is placed at other genomic sites (Kumar and Leffak, 1989), indicating that DNA sequences within the replicator can function to control the local chromatin environment (Figure 13A and B). Approximately 1500 bp upstream of the c-myc promoter P1 is a region of DNA termed a DNA unwinding element (DUE), due to its predicted helical instability. Levens and colleagues have shown this region to become single-stranded when the c-myc gene is expressed in vivo and in response to transcribing RNA polymerase *in vitro* (Kouzine et al, 2004). Levens termed the sequence the far-upstream sequence element (FUSE), and had subsequently shown that a protein called FBP (FUSE-binding protein) binds the region when it is single-stranded and functions to regulate c-myc transcription (Duncan et al, 1994; He et al, 2000; Michelotti et al, 1996). Thus in response to superhelical stress the DUE/FUSE sequence has the propensity to unwind both *in vitro* and *in vivo*, and thus may serve as an important regulatory element for the initiation of DNA replication.

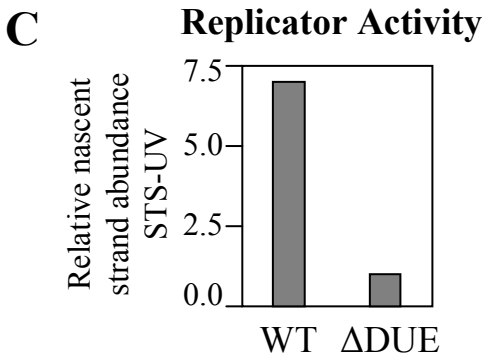
To examine the importance of the DUE in replicator function, our laboratory used the FLP-recombinase targeting system to integrate the c-myc replicator and various deletion mutants into the same genomic location on chromosome 18 in HeLa cells (Liu et al, 2003). A map of the integration site and inserted c-myc replicator is indicated in Figure 13B. Insertion of this sequence into this site imparts the production of short nascent strands from this chromosomal location. To examine the sequence elements of the replicator important for replication initiation from this site, several cell lines were created with unique c-myc replicator constructs inserted into the same chromosomal location. One such construct (ΔDUE, also known as Δ5) removes a 201 bp region of DNA that contains the DUE as well as three 10/11 matches to the budding yeast ARS-consensus sequence. As previously reported, replication origin activity at the ectopic c-myc replicator is significantly reduced in the ΔDUE cell line (Figure 13C).

Figure 13. A DNA unwinding element is required for the initiation of DNA synthesis from the c-myc replicator.

(A) Structural map and features of the replication origin upstream of the c-myc gene in human cells. Features including nuclease hypersensitive sites and a DUE are labeled with the approximate location within the replication origin. P_0, P_1, P_2 represent the three promoters for c-myc gene expression, and the black boxes represent c-myc exons. Relevant restriction enzyme sites are indicated (E, EcoRI; H, HindIII; X, XhoI), and c-myc replicator core stretches from the HindIII to XhoI sites (~2.4 kb) (B) Map of the c-myc replicator construct and integration cassette on chromosome 18 of the HeLa genome. The diamond represents the DUE. Sequence-tagged sites (STSs) commonly used for Q-PCR detection of newly synthesized DNA are shown with their approximate location. The recombination sites are labeled (FRT, FLP recombinanse target site) at their approximate locations, as are the drug resistance/sensitivity genes in the integrated cassette (Hyg, hygromycin; Neo, neomycin; TK, thymidine kinase). (C) Deletion of the DUE region from the c-myc replicator abolished origin activity. Nascent strand abundance was detected at STS-UV by Q-PCR amplification of 1-2 kb newly synthesized DNA prepared from clonal cell lines containing either the wild-type c-myc replicator or a mutant c-myc replicator with a 201 bp deletion that removes the proposed DUE. These data was obtained by Dr. Guoqi Liu and used with permission.


A
Human c-myc locus
bent
MNase HSS
DNase I HSS
DUE
ARS consensus
P0
P1
P2
E
H
X
E
Replicator fragment


B
Ectopic c-myc replicator
Hygr FRT
Neor
c-myc
FRT
TK
STS-Hyg
STS-UV
STS-pDV
STS-TK


C
Replicator Activity
Relative nascent strand abundance STS-UV
7.5
5.0
2.5
0.0
WT
ΔDUE

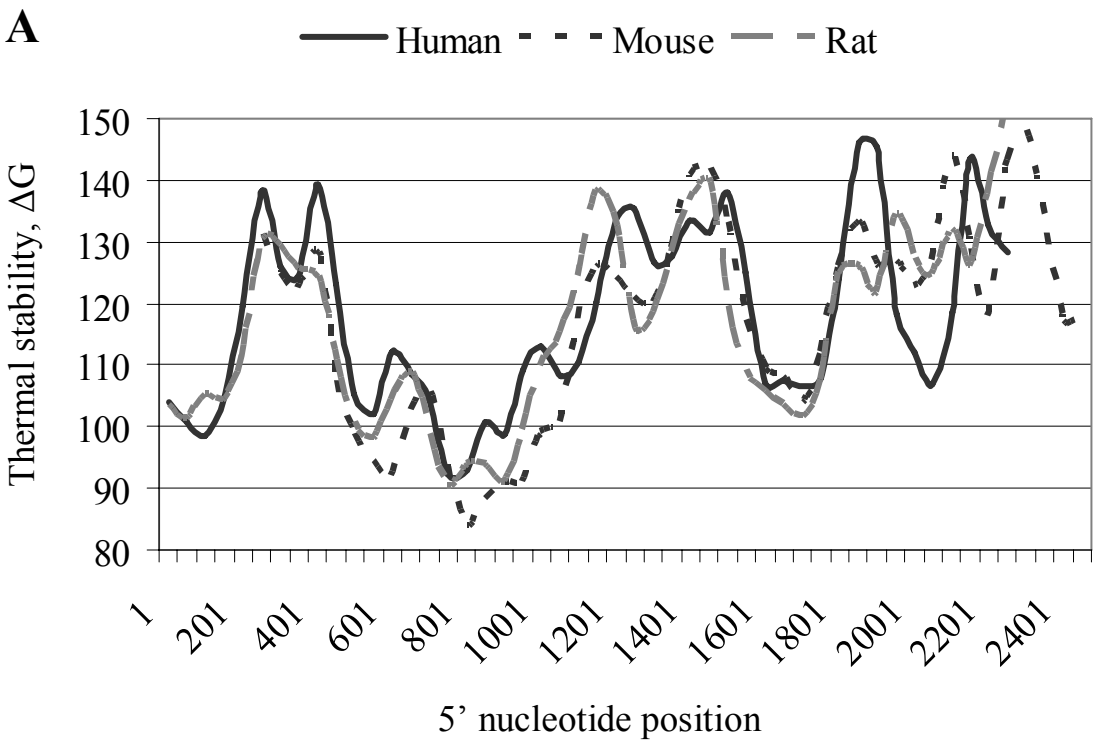
The c-myc DUE is conserved through evolution

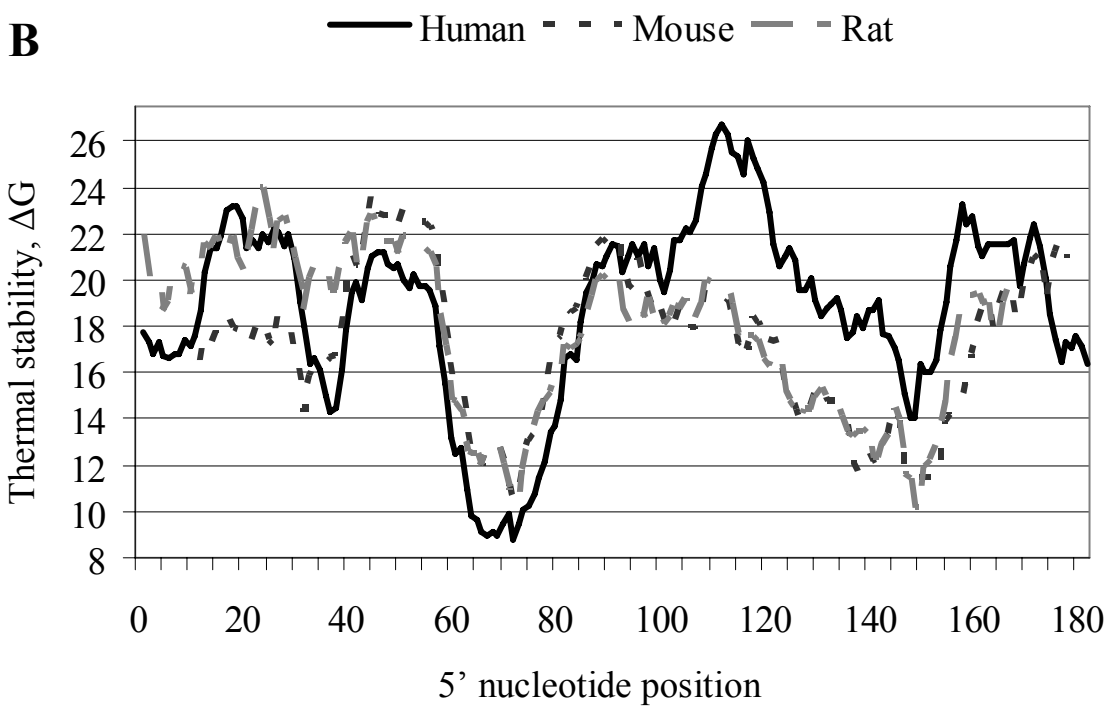
Although only a few replication origins have been identified or characterized in metazoan species, previous data has shown the c-myc replication origin to be conserved through evolution. It had been reported that chicken cells contain a replication origin upstream of the c-myc gene (Phi-van et al, 1998), and more recently it was shown that DNA replication initiates upstream of the c-myc gene in both somatic mouse and *Xenopus* cells (Girard-Reydet et al, 2004). The observation that replication initiates upstream of the c-myc gene in so many organisms suggests the presence of sequence elements upstream of the c-myc gene that are conserved through evolution, and also suggests that looking for sequence homology among these different species may be useful for the identification of important functional elements required for replicator activity. Since the DUE was shown to be important for replication origin activity in human cells, the DNA sequences of an approximately 2.4 kb HindIII/XhoI fragment was obtained from GenBank from the human, mouse, and rat genomes. Because of our interest in the function of helically unstable DNA sequences in replicator function, the sequences from all three organisms were analyzed by WEB-THERMODYN, an algorithm designed to predicted helical stability of DNA sequences. Interestingly the three sequences could be aligned quite well based simply on their intrinsic helical stability (Figure 14A). To examine the region of DNA deleted in the ΔDUE cell line more closely, the ~200 bp region comprising the region of the 2.4 kb sequences showing the lowest helical stability was further analyzed by WEB-THERMODYN using parameters that provided greater resolution. As shown in Figure 14B, there is a 30-40 bp region that shows a nearly identical thermal stability profile among the three organisms. This region corresponds to that of the DUE/FUSE, and sequence alignments showed 33 of the 38 nucleotides to be identical between human, mouse, and rat (data not shown). Together these results suggest that a region of helically unstable DNA is an evolutionarily conserved feature of the c-myc replication origin.

To examine whether the presence of the DUE may be important for the recruitment of specific protein factors to the replication origin, the c-myc DUE region was cloned upstream of a *HIS3* reporter gene in the budding yeast His^- strain YM4271 and then used to screen a Gal4AD-HeLa cDNA fusion protein expression library (Casper, 2004; Casper et al, 2005). One cDNA was isolated that supported rapid colony growth under selective conditions. The expressed protein was termed DUE-B, a designation for its

Figure 14. A region of DNA predicted to be helically unstable is an evolutionarily conserved feature of the HindIII/XhoI restriction fragment upstream of the c-myc gene.

(A) WEB-THERMODYN was used to predict the helical stability (thermal stability, ΔG) of c-myc HindIII/XhoI restriction fragments from human, mouse, and rat genomes. The free energy values were calculated over 100 bp windows (50 bp per step, 10 mM monovalent ion, 37°C). The region of lowest stability in all three species corresponds to the region deleted and analyzed for origin activity in the previous figure. (B) The 201 bp sequence that contains the human c-myc DUE, and deletion of which abolishes replicator activity, was similarly analyzed from the genomes of human, mouse, and rat. The free energy values were calculated over 20 bp windows (1 bp per step).


A
Human
Mouse
Rat
Thermal stability, ΔG
150
140
130
120
110
100
90
80
1
201
401
601
801
1001
1201
1401
1601
1801
2001
2201
2401
5' nucleotide position


B
Human
Mouse
Rat
Thermal stability, ΔG
26
24
22
20
18
16
14
12
10
8
0
20
40
60
80
100
120
140
160
180
5' nucleotide position

identification as a DNA-unwinding element binding protein. Northern blot analysis revealed a single mRNA species of ~1.4 kb, sufficient to encode the predicted 209 amino acids of DUE-B. Interestingly the amino acid sequence of DUE-B shows notable similarity to proteins found throughout bacteria and eukaryotes (Figure 15). The human sequence has been annotated in GenBank as human histidyl-aminoacyl-tRNA synthetase (Meng et al, 2002), although this designation has not been functionally validated. Identification and characterization of the yeast and bacterial homologs were first described at approximately the same time our laboratory initially cloned human DUE-B, and these proteins have been demonstrated to function as D-tyrosyl-tRNA^{Tyr} deacylases. Additional results indicated the yeast and bacterial proteins functioned as general D-aminoacyl-tRNA deacylases, and do not specifically act only on D-tyrosylated tRNAs. Interestingly Calendar and Berg had first described this enzymatic activity in 1967 in *E. coli* and found the activity also to be present in mammalian tissue extracts (Calendar and Berg, 1967). BLAST analyses with the human DUE-B sequence showed that the only protein sequences in mammalian and vertebrate genome databases to have significant sequence similarity to the bacterial proteins is the DUE-B sequence (data not shown), implying that human DUE-B is also a D-aminoacyl-tRNA deacylase. A unique feature of the vertebrate homologs though is an extension of ~60 amino acids at the C-terminus, suggesting that this domain may provide a unique, vertebrate-specific function for the DUE-B protein.

DUE-B is an abundant protein in *Xenopus* egg extracts and human cells

To examine the level of DUE-B protein expression in vertebrate replication systems, human cell and *Xenopus* egg extracts were analyzed by SDS-PAGE and western blotting. To examine the abundance of the protein in these systems, extract from a fixed amount number of HeLa cells or a fixed amount of *Xenopus* egg extract was analyzed by SDS-PAGE against known amounts of recombinant DUE-B (rDUE-B) from baculovirus-infected insect cells (Figure 16A). These data indicate DUE-B is expressed at ~4.25 ng per µl of egg extract (~180 nM), close to that of several other replication initiation proteins. The abundant store of replication proteins in *Xenopus* eggs is believed to contribute to the rapid 20 minute DNA synthesis phase of early development. In HeLa cells DUE-B was also observed to be expressed at high level, with 20-25 fg (5-6 x 10^5 molecules) of DUE-B protein per HeLa cell. Interestingly, the tetraploid HeLa cell genome (12 billion bp) is expected to have roughly 240,000 replication origins, assuming a

Figure 15. The human DUE-B sequence is conserved through evolution.

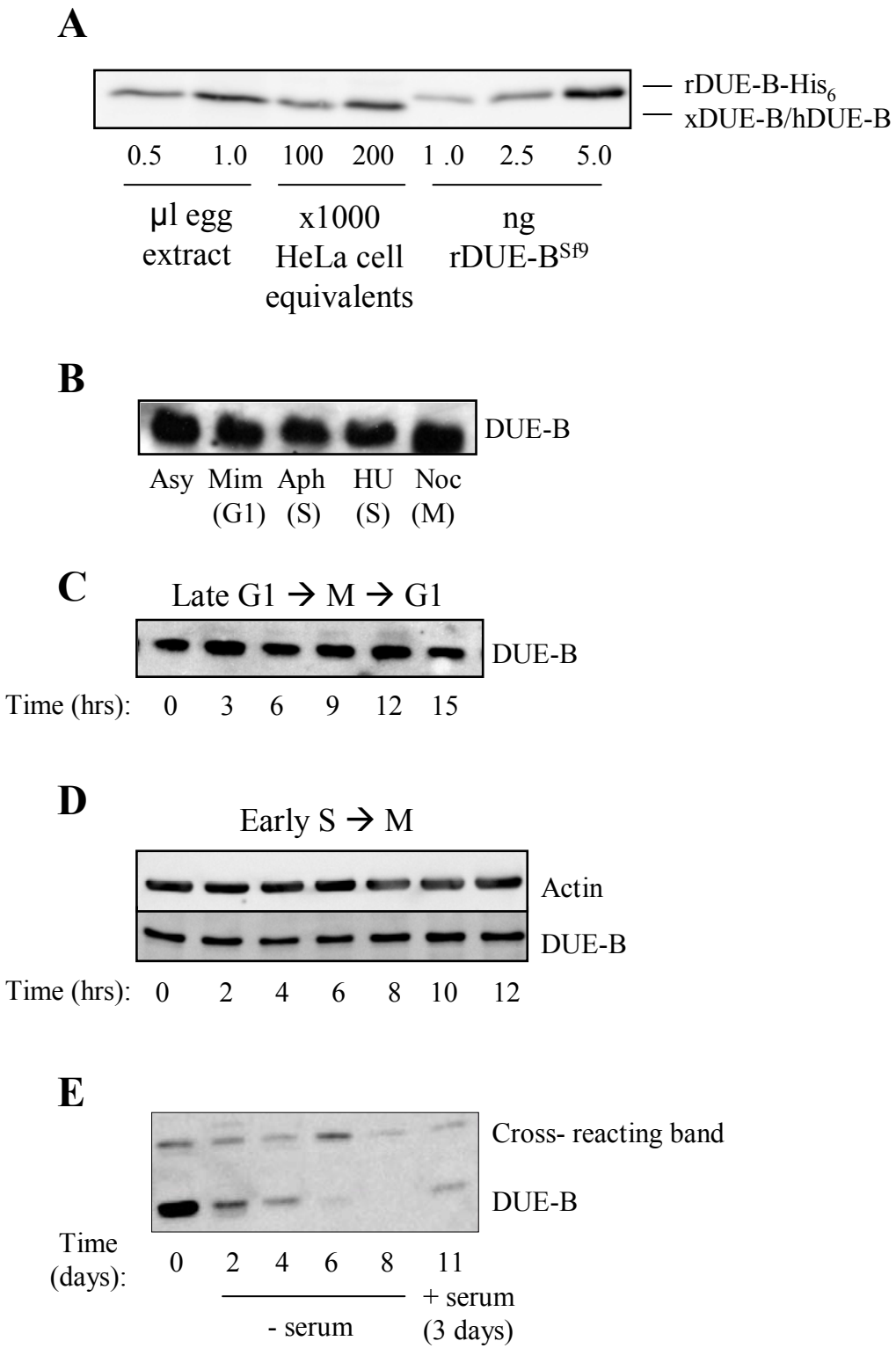
The DUE-B protein sequence was used to search for related proteins by sequence homology in the indicated organisms, and a CLUSTALW alignment was performed. Asterisks represent amino acids absolutely conserved throughout the indicated organisms, colons represent highly similar amino acids, and periods represent only partially conserved positions.

Multiple sequence alignment

```
Human           MKAVVQRVTRASVTVGGEQISAIGRGICVLLGISLEDTQKELEHMVRKILNLRVFEDES- 59
Mouse           MKAVVQRVTRASVTVGGEQISAIGRGICVLLGISMEDSQKELEHMVRKILNLRVFEDES- 59
Chicken         MKAIVQRVAQASVTVGGEQISSIGRGLCVLLGISLEDTQRELEHMVRKILNLRVFEDES- 59
Frog            MRAVIQRVTKASVTVGDEQISSIGRGICVLLGISVEDTQKDIEYMVRKILNLRLFTDES- 59
Zebrafish       MKAIIQRVTRASVTVGEEQISSIGRGLCVLLGISAEDTQKDVDYMVRKILNLRVFEDEN- 59
Pufferfish      MKAVVQRVVRASVCVGDEQVSSIGRGLCVLLGISAEDTQSDADYMIRKILNLRLFADEN- 59
Worm            MRVVLQRVTRAAVTVSDEVVGSIGRGLCVLVGIHRDDTEEDMKYIIRKILNLRIFPASE- 59
Fruitfly        MRAVIQRVKAAKVTVLDELVSSIGPGLCVLVGIKASDTAKDVEYLVRKILALRLFEEE-- 58
Arabidopsis     MRAVIQRVSSSSVTVDGRIVSEIGPGLLVLIGIHESDTESDADYICRKVLNMRLFSNETT 60
Fission         MKAVIQRVLNASVSVDDKIVSAIQQGYCILLGVGSDDTPEDVTKLSNKILKLKLFDN-A- 58
Budding         MKIVLQKVSQASVVVDSKVISSIKHGYMLLVGISIDDSMAEIDKLSKKVLSLRIFEDES- 59
E coli          MIALIQRVTRASVTVEGEVTGEIGAGLLVLLGVEKDDDEQKANRLCERVLGYRIFSDAE- 59
Hemophilis      MIALIQRVSQAKVDVKGETIGKIGKGLLVLLGVEKEDNREKADKLAEKVLNYRIFSDEN- 59
                *  ::*:*  : * *  .  . *  *  :*:*:  .*   .   : .::*  ::*

Human           GKHWSKSVMDKQYEILCVSQFTLQCVL-KGNKPDFHLAMPTEQAEGFYNSFLEQLRKTYR 118
Mouse           GKHWSKSVMDKEYEVLCVSQFTLQCVL-KGNKPDFHLAMPTEQAESFYNSFLEQLRKSYR 118
Chicken         GKHWSKSVMDKQYEVLCVSQFTLQCIL-KGNKPDYHMAMPTEQAESFYNNFLEQLRKAYK 118
Frog            GKPWCKSVMDKQYEVLCVSQFTLQCVL-KGNKPDYHMAMPSEQAEPFYNNFLQHMRKAYK 118
Zebrafish       GRAWSRSVMDGELEVLCVSQFTLQCLL-KGNKPDYHAAMPAELAQPFYNNMLEQLRETYK 118
Pufferfish      GRAWSKSVMDLDYEVLCVSQFTLQCML-KGNKPDFHAAMPAELAQPFYSHILENMRSIYK 118
Worm            EKPWDKSVMDLDLEVLSVSQFTLYGQF-KGNKLDFHTAMAPTEASKFYATFLESLKKAYK 118
Fruitfly        GKRWQKSVKDLNLELLCVSQFTLYHRL-KGNKPDFLAAMKGEEAQELYNQFLDRLGQSYD 117
Arabidopsis     GKGWDQNVMQRNYGVLLVSQFTLYGFL-KGNKPDFHVAMPPDKAKPFYASLVEKFQKAYN 119
Fission         EQPWKSTIADIQGEILCVSQFTLHARVNKGAKPDFHRSMKGPEAIELYEQVVKTLGESLG 118
Budding         RNLWKKNIKEANGEILSVSQFTLMAKTKKGTKPDFHLAQKGHIAKELYEEFLKLLRSDLG 119
E coli          -GKMNLNVQQAGGSVLVVSQFTLAADTERGMRPSFSKGASPDRAEALYDYFVERCRQQE- 117
Hemophilis      -DKMNLNVQQAQGELLIVSQFTLAADTQKGLRPSFSKGASPALANELYEYFIQKCAEK-- 116
                      .: :    :* ******     :* : .:  .     *  :*  .:.   .

Human           PELIKDGKFGAYMQVHIQNDGPVTIELESPAPGTATSDPKQLSKLEKQQQRKEKTRAKGP 178
Mouse           PELIRDGKFGAYMQVHIQNDGPVTIELESPAPGAASSDPKQLSKLEKQQQRKEKTRAKGP 178
Chicken         PELIKDGKFGAYMQVHIQNDGPVTIELESPA---AAVDPKQLAKLEKQQQRKEKTRTKVP 175
Frog            PELIKDGKFGAYMQLNIQNDGPVTIELEPPA---STADPKLLSKLEKQQQRKEKTRTKTQ 175
Zebrafish       PELIKDGQFGAKMQVLIQNDGPVTIQLESPP---APTDPKLLSKQEKQQQRKEKTRSKGP 175
Pufferfish      PEHIKDGKFGAKMQVNIQNDGPVTIELTSPS---APTDPKLISKQEKQQQRKEKTRSKGP 175
Worm            PEKIQDGKFAAMMSVDIVNDGPVTISFDSKE--------------------K-------- 150
Fruitfly        STKIK-----AYMQVHIENDGPVTINLESPE-------------------QKDTDR---- 149
Arabidopsis     PDAVKDGVFGAMMQVNLVNDGPVTMQLDSPQ----------------------------- 150
Fission         SDKIKKGVFGAMMNVQLVNNGPVTILYDTKE----------------------------- 149
Budding         EEKVKDGEFGAMMSCSLTNEGPVTIILDSDQ----------------------------- 150
E coli          -MNTQTGRFAADMQVSLVNDGPVTFWLQV------------------------------- 145
Hemophilis      -LPVSTGQFAADMQVSLTNDGPVTFWLNV------------------------------- 144
                          * *.  : *:****:

Human           SESSKERNTPR-KEDRSASSGAEGDVSSEREP 209
Mouse           SESSKERNAPR-KEDRSASSGAEGDVSSEREP 209
Chicken         SESSRERNAPRSKDDPSASSGAEGDVSSEREP 207
Frog            SESSREKSVPRSKDDPSASSGAEGDVSSEREP 207
Zebrafish       SDSSREKAAQRSKVDPSASSGAEGDVSSEREP 207
Pufferfish      SESSK-KSGQWHRREPNTSSGAEGDVSSEKE- 205
Worm            --------------------------------
Fruitfly        -EVDK--------------------------- 153
Arabidopsis     --STK--------------------------- 153
Fission         --------------------------------
Budding         --------------------------------
E coli          --------------------------------
Hemophilis      --------------------------------
```

Figure 16. DUE-B is an abundant protein in Xenopus egg extracts and human cells, is expressed throughout the cell cycle, and its expression is stimulated by serum.

(A) DUE-B is expressed at a high level in Xenopus egg extracts and in HeLa cells. Xenopus egg extract and HeLa cell extract was analyzed by SDS-PAGE and western blotting against a range of amounts of recombinant DUE-B purified from baculovirus-infected insect cells. (B) The level of DUE-B expression in HeLa cells arrested at various phases of the cell cycle was determined by western blotting (Asy, asynchronous;y growing cells; Mim, 500 μM mimosine for 24 hours; Aph, 1 μg/ml aphidicolin for 24 hours; HU, 2 mM hydroxyurea for 24 hours; Noc, 100 ng/ml nocodazole for 24 hours). (C) DUE-B expression is not significantly altered in cells progressing through S phase. HeLa cells were arrested in late G1 by treatment with 500 μM mimosine for 24 hours, then released into fresh medium for the indicated length of time. Cells were harvested, whole cell extracts prepared, then analyzed by SDS-PAGE and western blotting. Cells reach mitosis by 12 to 14 hours, then begin to enter a new G1 phase. (D) DUE-B protein expression does not significantly change as cells progress through S phase into mitosis. HeLa cells were arrested for with aphidicolin (1 μg/ml) for 18 hours then released into fresh medium containing 100 ng/ml nocodazole to stop cells from moving through to G1 phase. At the indicated time points, cells were harvested, lysed in T-PER (Pierce) containing both a protease and phosphatase inhibitor cocktail (Pierce), and analyzed by western blotting. Movement of cells through S phase was monitored by flow cytometry, and indicated that all the cells had reached mitosis by 8 hours after release from the aphidicolin block. (E) DUE-B protein expression is affected by serum stimulation. Asynchronously growing HeLa cells were grown in medium lacking serum for the indicated number of days. At day 8, the medium from one plate of cells was replaced with DMEM containing 10% newborn calf serum and grown for 3 more days. On the indicated days, whole cell extracts were prepared and 20 μg of protein was loaded on an SDS-PAGE gel for western blotting. The cross-reacting band is sometimes observed with the anti-DUE-B antiserum, and was used for normalization purposes.


A
rDUE-B-His6
xDUE-B/hDUE-B
0.5
1.0
100
200
1 .0
2.5
5.0
µl egg extract
x1000 HeLa cell equivalents
ng rDUE-BSf9
B
DUE-B
Asy
Mim (G1)
Aph (S)
HU (S)
Noc (M)
C
Late G1 → M → G1
DUE-B
Time (hrs): 0 3 6 9 12 15
D
Early S → M
Actin
DUE-B
Time (hrs): 0 2 4 6 8 10 12
E
Cross- reacting band
DUE-B
Time (days):
0 2 4 6 8 11
- serum
+ serum (3 days)

replicon size of 50 kb. Thus there are approximately 2 DUE-B molecules, or 1 DUE-B dimer (see structural characterization below) per replication origin.

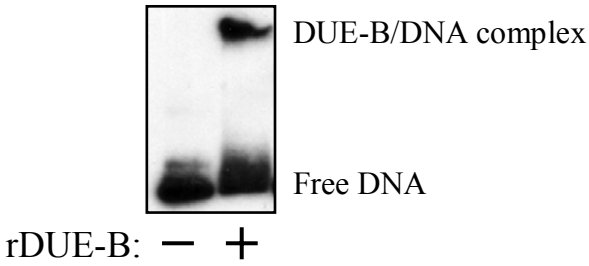
To examine whether DUE-B protein levels are regulated in a cell cycle-dependent fashion, HeLa cells were arrested at various stages of the cell cycle and then harvested for western blot analysis using anti-DUE-B antiserum. As indicated in Figure 16B, DUE-B protein levels remain relatively stable at all phases of the cell cycle, although slightly more protein was visible in mitotic cells. Since the chemical reagents employed might not represent true cell cycle regulation, HeLa cells were arrested in either late G1 with mimosine (Figure 16C) or in early S phase with aphidicolin (Figure 16D), and then released from the block into fresh medium to follow the cells through S phase. Again, DUE-B protein levels remain largely unchanged, though there appears to be a slight increase in DUE-B levels as cells progress through S phase, suggesting DUE-B protein expression may be regulated in an S-phase dependent manner. The abundance of DUE-B and its identification as a replication origin binding protein suggested in might be important for some aspect of cell proliferation or growth. To determine if DUE-B expression is affected by the cell proliferation state, the culture medium of asynchronously growing HeLa cells was replaced with medium lacking serum. DUE-B protein levels decreased significantly within two days of serum withdrawal, suggesting that DUE-B expression is serum-dependent. After 8 days without serum, DUE-B expression was completely eliminated, but could then be restored by adding serum back to the culture medium. Together these results suggest that the DUE-B protein expression is stable throughout the cell cycle and that DUE-B expression is inhibited in non-proliferating HeLa cells.

DUE-B binds DNA *in vitro* and *in vivo*

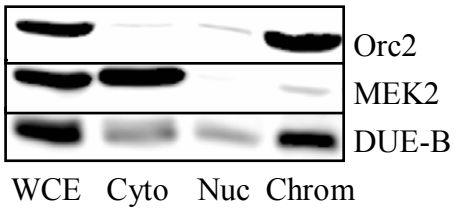
The identification of DUE-B in the yeast one-hybrid assay as a DUE-binding protein suggested DUE-B has the ability to bind to DNA. *In vitro* rDUE-B bound a dsDNA PCR product (Figure 17A), suggesting an intrinsic affinity for DNA. To examine whether DUE-B bound DNA or was localized on DNA *in vivo*, HeLa cells were fractionated to enrich for chromatin-bound proteins. This method successfully separated cytosolic proteins (MEK2) from proteins reported to be exclusively bound to chromatin, such as Orc2. As shown in Figure 17B, although DUE-B appeared in both the soluble cytosolic and soluble nuclear fractions, most of the protein was in the chromatin-enriched fraction. Immunofluorescence experiments yielded similar results (Katrangi and Leffak, unpublished; Casper et al,

Figure 17. DUE-B binds DNA *in vitro* and binds chromatin in HeLa cells and Xenopus egg extracts. (A) DUE-B binds DNA *in vitro*. Recombinant DUE-B from baculovirus-infected insect cells was incubated in the absence or presence of a 123 bp ^{32}P-labeled PCR product from the c-myc replicator and electrophoresed on a 4% native acrylamide gel. (B) DUE-B is bound on the chromatin fraction of HeLa cells. HeLa cells were fractionated to yield a soluble cytoplasmic fraction, a soluble nuclear fraction, and a chromatin-enriched fraction. Aliquots of each fraction from an equivalent number of cells were run with whole cell extract (WCE) on SDS-PAGE and then analyzed by western blotting with antibodies against the indicated proteins. (C) Xenopus sperm chromatin was added to a low-speed interphase egg extract at a final concentration of 1800/µl. After incubation for 15 minutes at room temperature, the reaction was diluted 10-fold in cold buffer XB containing 0.05% Triton X-100 on ice for two minutes and layered over a 0.7 M sucrose cushion made in XB. The chromatin was then pelleted at 3900 x g for 5 minutes at 4°C, washed once in buffer XB, and re-centrifuged. Proteins bound to the chromatin were eluted in SDS-PAGE sample buffer.

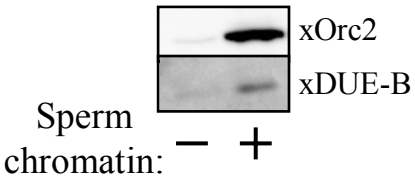
A


DUE-B/DNA complex
Free DNA
rDUE-B: – +

B


Orc2
MEK2
DUE-B
WCE Cyto Nuc Chrom

C


xOrc2
xDUE-B
Sperm chromatin: – +

2005). Additional experiments demonstrated that the binding to chromatin may be weak because DUE-B can be washed off the chromatin with moderate salt or non-ionic detergent (data not shown). To examine whether DUE-B binds DNA in another system, a low-speed *Xenopus* egg extract was incubated in the absence or presence of sperm chromatin for 15 minutes, then fractionated through sucrose and analyzed by western blotting. xDUE-B appeared along with xOrc2 on the chromatin under these conditions (Figure 17C), suggesting DUE-B binding to DNA is physiological.

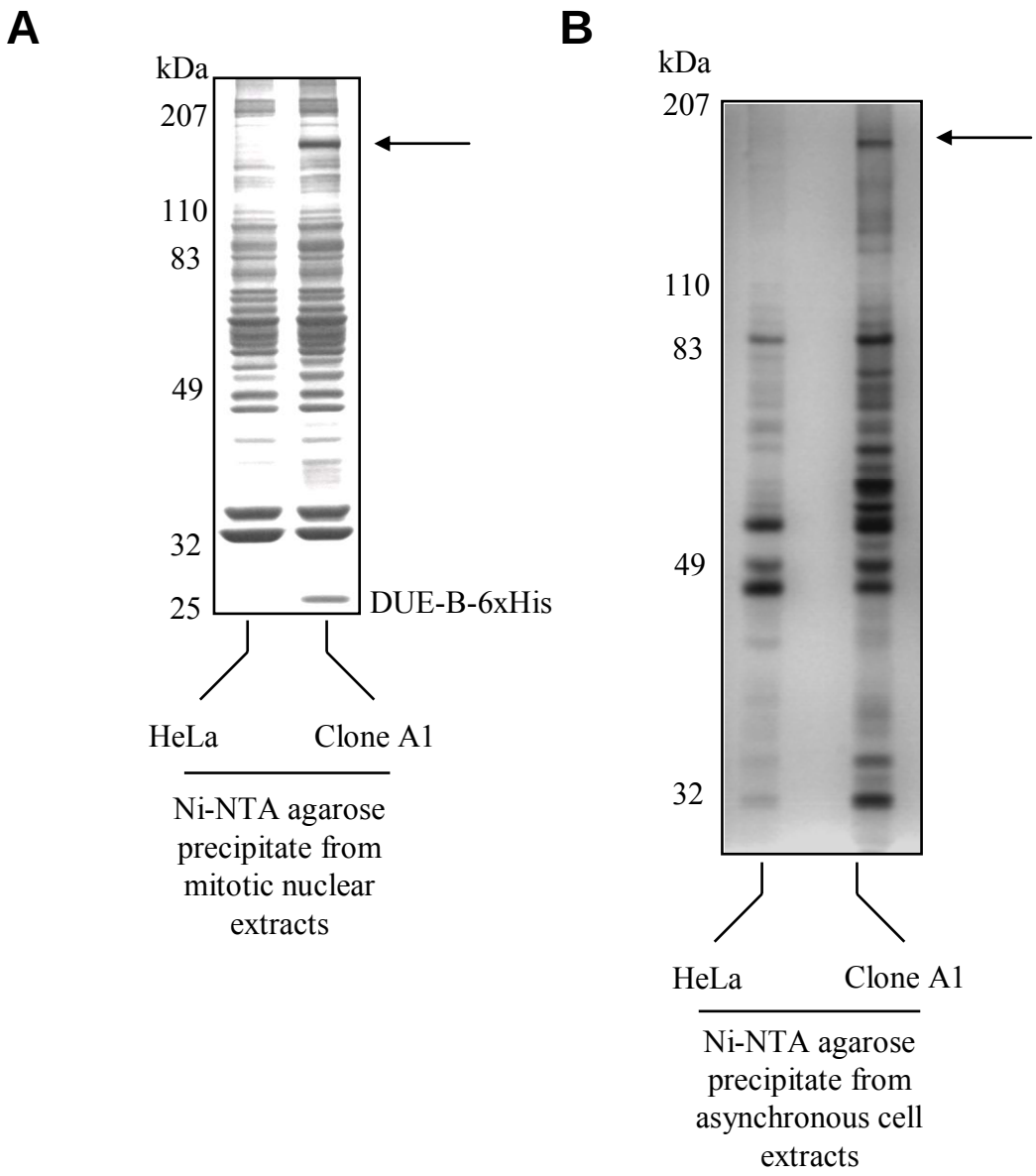
Further *in vivo* studies by our laboratory showed DUE-B to be localized to the c-myc origin and the lamin B2 origin (Ghosh et al, 2006). At the c-myc replication origin, the distribution of DUE-B throughout the locus showed it to be enriched at or near the DUE in asynchronous cells or in cells arrested in late G1 phase. In mitotic cells, DUE-B was still bound to chromatin, but became more distributed throughout the locus. The requirement for the DUE in DUE-B binding to the replicator was studied using clonal cells containing the wild-type or mutant c-myc constructs integrated at the same ectopic location on chromosome 18. Deletion of 201 bp containing the DUE abolished both replicator function (Figure 13C, (Liu et al, 2003)) and DUE-B binding to the replicator (Ghosh et al, 2006). The binding of Orc2 and Mcm3 to the c-myc replicator were not affected in the mutant (ΔDUE) cell line. Interestingly, recent data from our laboratory demonstrates that other helically unstable DNA sequences can replace the endogenous DUE sequence, both in the ability to restore function to the replicator and to restore DUE-B binding to the replicator (Liu, Ghosh and Leffak, unpublished). These results suggest that DUE-B recruitment to replication origins is not dependent upon a particular DNA sequence, but rather on the presence of helically unstable DNA that restores replicator functionality. Whether DUE-B recognizes the DUE directly or another structural aspect of the replication origin remains to be determined.

DUE-B interacts with protein components of the replication machinery

The presence of DUE-B at replication origins suggests it may interact with other proteins that are involved in replication initiation. Co-immunoprecipitation experiments from either *Xenopus* egg extracts or human cell extracts failed to detect interactions with members of the ORC or MCM complexes (data not shown), suggesting that DUE-B may interact with replication proteins after the activation of pre-RCs. Several approaches have been used to try to identify interacting proteins in both human cells and in *Xenopus* egg extracts. Extracts from a clonal HeLa cell line expressing a 6xHis-tagged DUE-B cDNA was

Figure 18. DUE-B interacts with protein(s) of 180 kDa molecular weight in human cells.

(A) HeLa cells or a clonal HeLa cell line stably expressing a cDNA encoding a His-tagged cDNA of DUE-B (referred to as Clone A1 cells) arrested in mitosis with nocodazole, then lysed in using Buffer A (Vashee et al, 2001; Casper, 2004) containing 0.1% Tween-20. An equivalent amount of total protein was incubated with Ni-NTA agarose and affinity-purified proteins were eluted with SDS-PAGE sample buffer, electrophoresed on an SDS-PAGE gel, and finally stained with silver. The arrow indicates one or more protein(s) of ~180 kDa that co-precipitates with the His-tagged DUE-B. (B) HeLa cells and Clone A1 cells were lysed by Dounce homogenization in egg lysis buffer to extract cellular proteins. The extract was then incubated with Ni-NTA agarose and affinity purified proteins separated by SDS-PAGE. The gel was stained with silver, and an ~180 kDa band is indicated by an arrow.

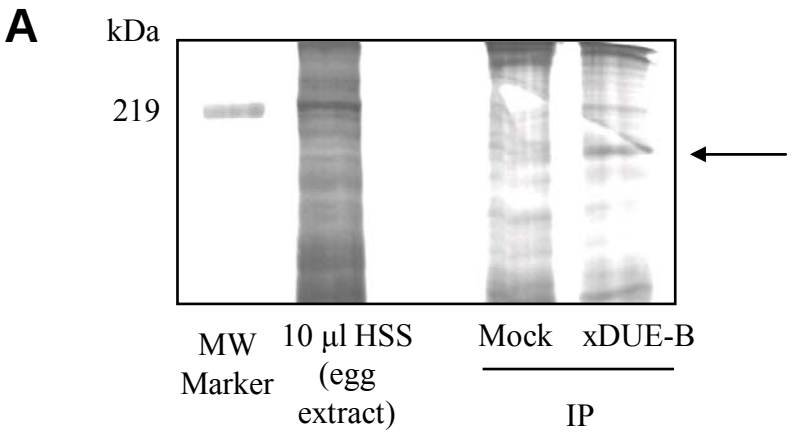

A
kDa
207
110
83
49
32
25
DUE-B-6xHis
HeLa
Clone A1
Ni-NTA agarose precipitate from mitotic nuclear extracts
B
kDa
207
110
83
49
32
HeLa
Clone A1
Ni-NTA agarose precipitate from asynchronous cell extracts

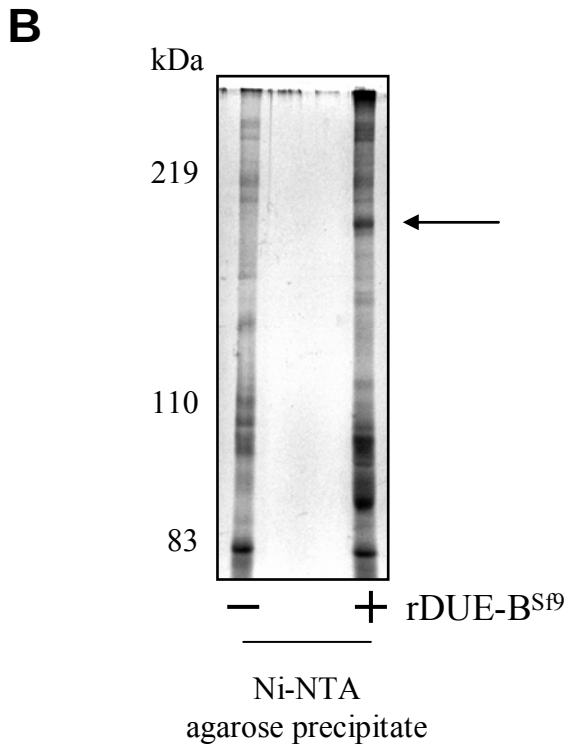
used with Ni-NTA agarose chromatography to purify DUE-B interacting proteins. Displayed in Figure 18, one or more proteins of ~180 kDa in molecular weight could be co-isolated with His-tagged DUE-B in both mitotic (Figure 18A) and asynchronous cells (Figure 18B). Interestingly, proteins of the same molecular weight could be co-precipitated with xDUE-B by immunoprecipitation from *Xenopus* egg extract (Figure 19A) or by Ni-NTA agarose precipitation of recombinant 6xHis-tagged DUE-B pre-incubated in egg extract (Figure 19B). These results suggest that DUE-B may interact with the same homologous proteins in both human cells and in *Xenopus* egg extracts.

To identify the proteins comprising this ~180 kDa band, a scaled-up experiment was performed in which xDUE-B was immunoprecipitated from 1.5 ml of a high-speed *Xenopus* egg extract. The coomassie-stained band was digested in-gel with trypsin and then analyzed by MALDI-TOF mass spectrometry. The 84 peptide masses obtained were analyzed using the MASCOT peptide mass fingerprint algorithm. Although it is often difficult to accurately identify large proteins by peptide mass fingerprinting alone, several potential interesting proteins were obtained in the searches that were performed. As displayed in Table 2, several replication or repair proteins had a significant number of predicted tryptic peptide masses that matched those found in the MALDI-TOF analysis, including TopBP1/Cut5/Mus101 (13-16 matches), DNA polymerase alpha (21 matches), DNA polymerase epsilon (17 matches), DNA methyltransferase (18 matches), BRCA1 (16 matches), and 53BP1 (16 matches). Since all of these proteins are ~180 kDa in molecular weight, and many have been reported in the literature to interact with one another, the difficulty in positively identifying the components of the 180 kDa band was further enhanced. To determine whether xDUE-B interacts with any of these proteins, antibodies against xMus101, xPolε, and xPolα were obtained and used in western blots of xDUE-B immunoprecipitates. Unfortunately, none gave an indication that the protein interacted with xDUE-B (data not shown).

Gel filtration analyses of recombinant DUE-B purified from baculovirus-infected insect cells and incubated in a *Xenopus* egg extract suggested a shift of rDUE-B from its normal state as a dimer to that of a high molecular weight complex, indicating an interaction with other proteins in the extract. Ni-NTA agarose-mediated pull-down of rDUE-B^{Sf9} incubated in egg extracts showed the co-purification of a 180 kDa protein (Figure 19B), and in several experiments a 70 kDa protein as well. A scaled up experiment in

Figure 19. xDUE-B and rDUE-B^{Sf9} interact with protein(s) of ~180 kDa in Xenopus egg extracts. (A) xDUE-B was immunoprecipitated from 100 µl high-speed, Xenopus interphase egg extract with anti-DUE-B antiserum or normal rabbit serum. The immunoprecipitate was electrophoresed on an 8% polyacrylamide gel then stained with silver. The position of the 180 kDa interacting protein is indicated with an arrow. (B) rDUE-B^{Sf9} (6 µg) was added to 100 µl of a high-speed, Xenopus interphase egg extract and then affinity purified with Ni-NTA agarose. Co-precipitated proteins were separated by SDS-PAGE and the gel was stained with silver. The position of the ~180 kDa co-precipitating protein is indicated with an arrow.


A
kDa
219
MW
Marker
10 µl HSS
(egg
extract)
Mock
xDUE-B
IP


B
kDa
219
110
83
–
+
rDUE-B^{Sf9}
Ni-NTA
agarose precipitate

which rDUE-B^{Sf9} was pulled down from 2 ml of high-speed *Xenopus* egg extract yielded a coomassie-stainable 70 kDa band that was excised and analyzed by tandem MS/MS analysis. Several high-confidence peptide sequences were obtained from the proteins listed in Table 3. Interestingly, a peptide from xDnmt1 was found by this analysis, as was similarly suggested from MALDI-TOF analysis (Table 2). The relevance of the other proteins discovered in this experiment is currently unclear, though all have been implicated in cytokinesis. Interestingly many of the proteins involved in replication initiation at origins have also been shown to have roles in chromosome maintenance, centrosome duplication, and cytokinesis (Stillman, 2005).

Preliminary evidence for Dnmt1 as the 180 kDa-DUE-B-interacting proteins was confirmed using antibodies that recognize xDnmt1 or hDnmt1. xDUE-B immunoprecipitated from *Xenopus* egg extracts showed co-immunoprecipitation of xDnmt1 (Figure 20A) using an anti-xDnmt1 antibody. Similarly, Dnmt1 was found to co-purify with His-tagged DUE-B by Ni-NTA agarose affinity chromatography from a HeLa cell line stably expressing 6xHis-tagged DUE-B (Figure 20B). In both cases a very limited amount of the total Dnmt1 present in the extracts co-purified with DUE-B. Similar results have since been obtained by using Ni-NTA agarose pull-downs with rDUE-B^{Sf9} incubated in either *Xenopus* egg extract or HeLa nuclear extract (Katrangi and Leffak, unpublished), suggesting the interaction occurs with both endogenous and recombinant/exogenous forms of DUE-B.

Since many of the protein components of the replication machinery interact with one another and function together at the replication fork, we were interested in whether DUE-B might also be a component of the multi-protein replication complex termed the DNA synthesome (Abdel-Aziz et al, 2003; Jiang et al, 2002). This biochemically purified complex was shown to contain >40 replication and repair proteins that remain as a complex after several biochemical manipulations, including after native polyacrylamide gel electrophoresis (Tom et al, 1996). An outline of the purification strategy is provided in Figure 21A. To determine whether DUE-B may be a component of the DNA synthesome, fractions from the different stages of purification were obtained from Dr. Linda Malkas, run on SDS-PAGE, and analyzed by western blotting. Both the P4 and Q-sepharose elution fractions contain significant enrichment for DNA polymerase activity. By western blot analysis, DUE-B was found to co-purify with the replication-competent fractions

Table 2. Summary of MALDI-TOF analysis of ~180 kDa xDUE-B interacting protein.

MALDI-TOF analysis of a coomassie stained 180 kDa band co-immunoprecipitated with xDUE-B from Xenopus egg extacts was performed at the Ohio State University Proteomics Facility and yielded 84 peptide masses. The proteins listed are ranked according their MOWSE score based on an analysis using a Matrix Biosciences algorithm. Proteins in bold represent proteins of ~180 kDa known to function in DNA replication, repair, or checkpoint regulation.

Rank	MOWSE Score	#/84 matches	Protein MW (kDa)	Protein	Function
1	47411	20	176.7	Roundabout-1	Axon guidance
2	12300	16	169.2	**Cut5-related protein**	Replication initiation/checkpoints
3	5007	17	187.6	**DNA polymerase epsilon**	DNA replication
4	4493	21	165.0	**DNA polymerase alpha catalytic subunit**	DNA replication
5	3005	16	180.3	RNA polymerase I large subunit	Transcription
6	2853	13	169.1	**Mus101**	Replication initiation/checkpoints
7	2160	15	180.6	Lipoprotein receptor-related protein 6	
8	1843	22	186.7	MGC68772 protein (similar to Rho/Rac GEF)	Actin cyto/nuclear transport/cell div
9	1513	10	179.7	Lipoprotein receptor-related protein 5	
10	905	17	184.8	IQ motif containing GTPase activating protein 1	Actin cytoskeleton
11	841	17	170.2	LOC398587 (plectin/alpha actinin)	Actin cytoskeleton/crosslinker
12	549	18	168.0	**DNA (cytosine-5-)-methyltransferase**	DNA replication
13	476	10	164.4	MGC68875 (alpha2-macroglobulin)	
14	125	12	189.7	IQ motif containing GTPase activating protein 2	Actin cytoskeletion
15	110	16	175.7	**BRCA1**	DNA damage/checkpoints
16	53.1	12	168.4	Kinesin-like protein	Actin cytoskeleton motor protein
17	21.7	16	190.7	**53BP1**	DNA damage/checkpoints

Table 3. Proteins identified by MS/MS as interacting with rDUE-B incubated in Xenopus egg extracts.

A coomassie-stained 70 kDa band that co-purified with recombinant DUE-B (baculovirus-expressed) after incubation and pull-down with Ni-NTA agarose from Xenopus egg extracts was analyzed by tandem mass spectrometric analysis at the Ohio State University Proteomics Facility. Several peptides from the listed proteins were found. Asterisk denotes that the protein was found by Skop et al (Science, 2004) in a proteomic analysis of the HeLa cell mid-body.

Protein	**Function(s)**	**Implicated in cytokinesis?**
Cingulin	Cell adhesion/tight junction biogenesis	Fesenko et al, Mech of Dev 2000
Clathrin heavy chain*	Endocytosis, vesicle transport	Feng et al, Exp Cell Res 2002 Gerald et al, Cell Motil and Cytoskel 2001 Niswonger et al, PNAS 1997 Skop et al, Science 2004
Dynamin-like protein*	Endocytosis	Skop et al, Science 2004 Thompson et al, Current Biology 2002
Heat shock cognate protein 70kDa*	Many	Skop et al, Science 2004
DNA (cytosine-5-)-methyltransferase* (dnmt1)	DNA replication/imprinting	Skop et al, Science 2004

Figure 20. DUE-B interacts with Dnmt1 in Xenopus egg extracts and human cells.

(A) xDUE-B was immunoprecipitated from 100 µl of a Xenopus high-speed egg extract, run on an 8% SDS-polyacrylamide gel and probed for xDnmt1 by western blot. Lane labeled HSS represents the amount of xDnmt1 in 1 µl of extract (1% input). (B) WCE (1 mg) made as in Figure 30B from HeLa cells and a HeLa cell line (termed Clone A1) stably expressing a His-tagged DUE-B cDNA were incubated with Ni-NTA agarose. The affinity-precipitated material was separated by SDS-PAGE and western blotted with an antibody against hDnmt1. Baculovirus expressed rDUE-B has also been shown to interact with Dnmt1 in both Xenopus egg extracts and a HeLa nuclear extract (Katrangi and Leffak, unpublished data).

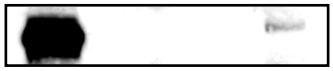
A

xDnmt1

HSS NRS Anti-DUE-B

Immunoprecipitate

B



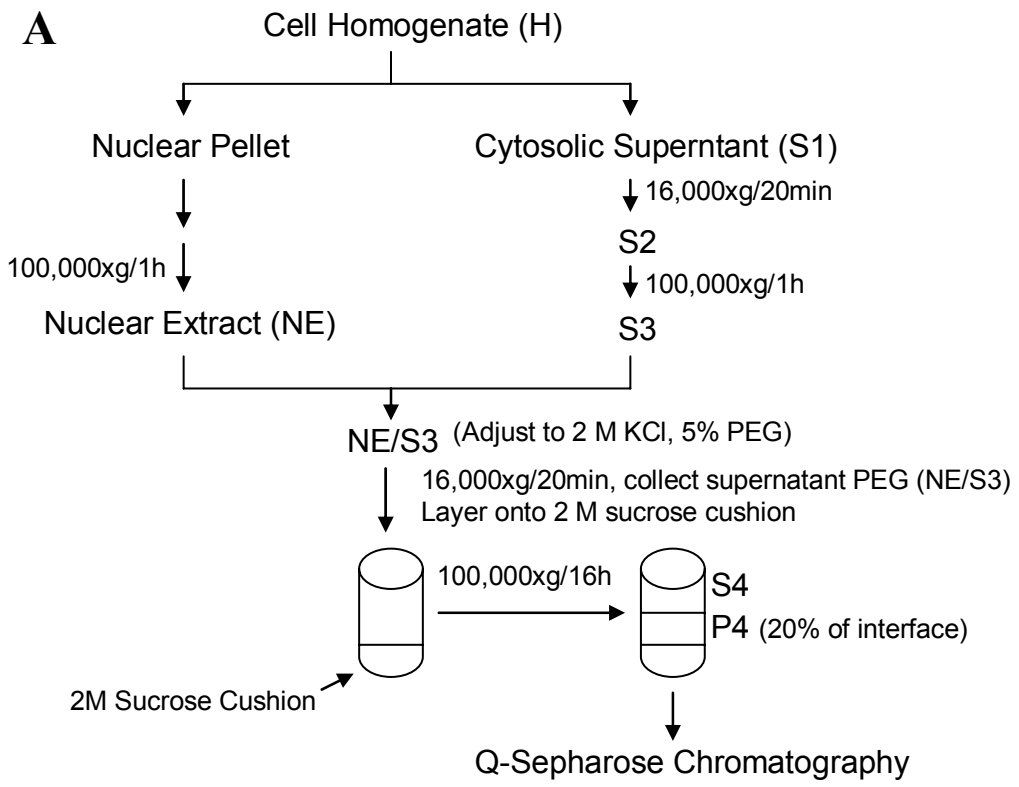
hDnmt1

WCE HeLa Clone A1

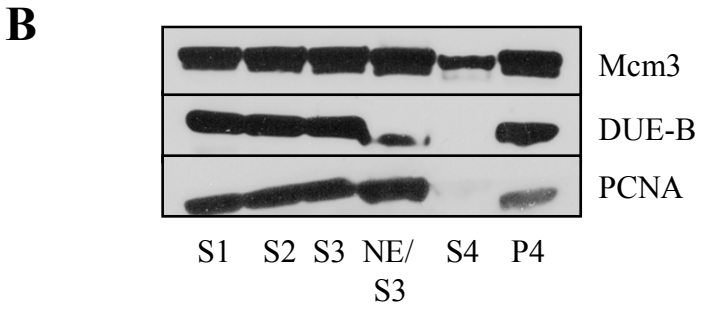
Ni-NTA Pull-down

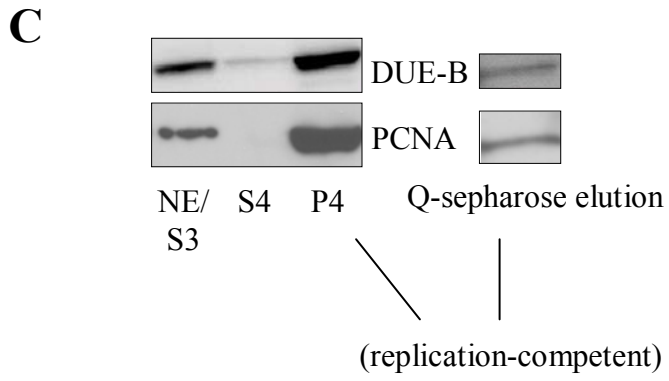
Figure 21. DUE-B is a component of the DNA synthesome.

A biochemically purified multi-protein replication complex termed the DNA synthesome was obtained from Linda Malkas (IUPUI). The subcellular fractionation scheme is displayed in (A). Aliquots of various fractions were analyzed by SDS-PAGE and western blotting to detect the presence of the indicated proteins. Fractions from HeLa cells (A) and IMR-32 neuroblastoma cells (B) demonstrate the presence of DUE-B in the replication competent P4 fraction and in the replication-competent Q-sepharose chromatographic fraction. In (C), the signal for PCNA and DUE-B in the Q-fraction sample was significantly weaker than in the neighboring lane, so a separate, longer exposure was used to visualize these proteins in this fraction.



Flow diagram of subcellular fractionation scheme





in both HeLa cells (Figure 21B) and the neuroblastoma cell line IMR-32 (Figure 21C). Interestingly, Dnmt1 has also been reported to be a component of the DNA synthesome (Vertino et al, 2002).

Together these results suggest that DUE-B interacts with the protein machinery involved in the replication of chromosomal DNA. Whether DUE-B is involved in the recruitment or regulation of any of these proteins at replication origins is currently unclear.

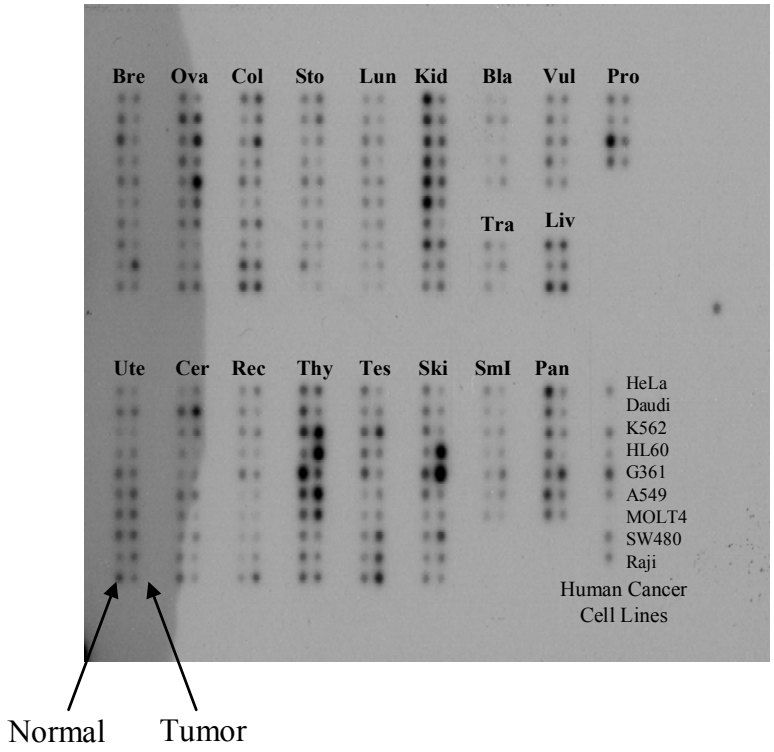
DUE-B expression is elevated in tumors

Several replication initiation proteins have emerged in recent years as potential diagnostic markers of tumor progression (Dudderidge et al, 2005; Gonzalez et al, 2003; Gonzalez et al, 2004; Gonzalez et al, 2005; Wohlschlegel et al, 2002), or have been shown to be overexpressed in subsets of cancer ((Karakaidos et al, 2004). Since DUE-B is an abundant protein in proliferating HeLa cells and is a component of the replication machinery, we were interested in whether DUE-B expression might be correlated with the enhanced proliferation potential of the transformed, tumorigenic phenotype of cancer cells. We obtained a Cancer Profiling Array II that contained cDNA from 154 tumor and corresponding normal tissues from individual patients. The array was probed first with a ^{32}P-labeled DUE-B cDNA probe (Figure 22A) and then stripped and re-hybridized with a ^{32}P-labeled ubiquitin cDNA probe (Figure 22B) to determine the relative expression of DUE-B mRNA in tumor tissue versus normal tissue. The data are quantitated in Figure 23, where the DUE-B to ubiquitin signals in the tumor tissue was divided by the DUE-B to ubiquitin signals in normal tissue to obtain the relative expression of DUE-B mRNA in tumor tissue to that found in normal tissue. A ratio greater than 1 indicated DUE-B expression is elevated in the tumor sample, whereas a ratio less than 1 indicated DUE-B expression was decreased in the tumor sample relative to that in normal tissue. As can be seen in Figure 23, DUE-B expression was increased in ~80% of the samples overall, albeit to a variable degree among the different samples. Several tissue types, such as ovary and skin, show DUE-B expression to be increased in nearly all the samples for that particular tissue type. DUE-B mRNA expression was elevated an average of 1.8-fold among all the tumor samples, and in some individual samples DUE-B was increased 4- to 8-fold. These results suggest that tumor progression may be correlated with DUE-B mRNA or protein levels, and may make DUE-B a useful diagnostic marker for the state of tumor progression.

Figure 22. DUE-B expression is elevated in many tumors.

A Cancer Profiling Array was obtained from BD Biosciences and contains amplified cDNA from 154 tumor and corresponding normal tissues from individual patients. Samples from individual patients are matched, such that in each pair the sample on the left is from normal tissue and the sample on the right is from tumor tissue. The array also contains cDNA from nine cancer cell lines and hybridization controls. The array was hybridized first with a ^{32}P-labeled probe against the DUE-B cDNA (A), then stripped and reprobed for ubiquitin to normalize DUE-B expression (B). The hybridizations were performed by Gia Randall, and the analyses performed by myself. The array was scanned by a phosphorimager and also exposed to film. The tissue types are abbreviated as follows: Bre (breast), Ova (ovary), Col (colon), Sto (stomach), Lun (Lung), Kid (kidney), Bla (bladder), Tra (trachea), Vul (vulva), Liv (liver), Pro (prostate), Ute (uterus), Cer (cervix), Rec (rectum), Thy (thymus), Tes (testis), Ski (skin), SmI (small intestine), Pan (pancreas).

A


Bre
Ova
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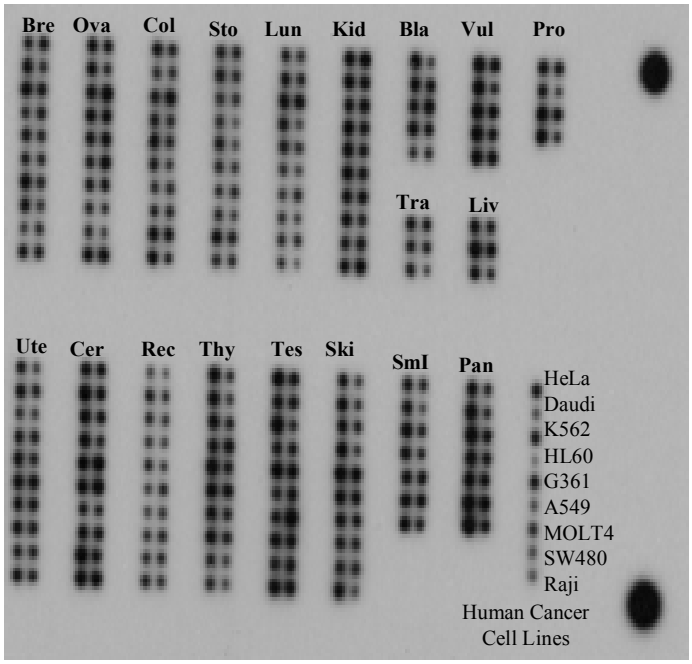
Cer
Rec
Thy
Tes
Ski
SmI
Pan
HeLa
Daudi
K562
HL60
G361
A549
MOLT4
SW480
Raji
Human Cancer
Cell Lines
Normal
Tumor

B


Bre
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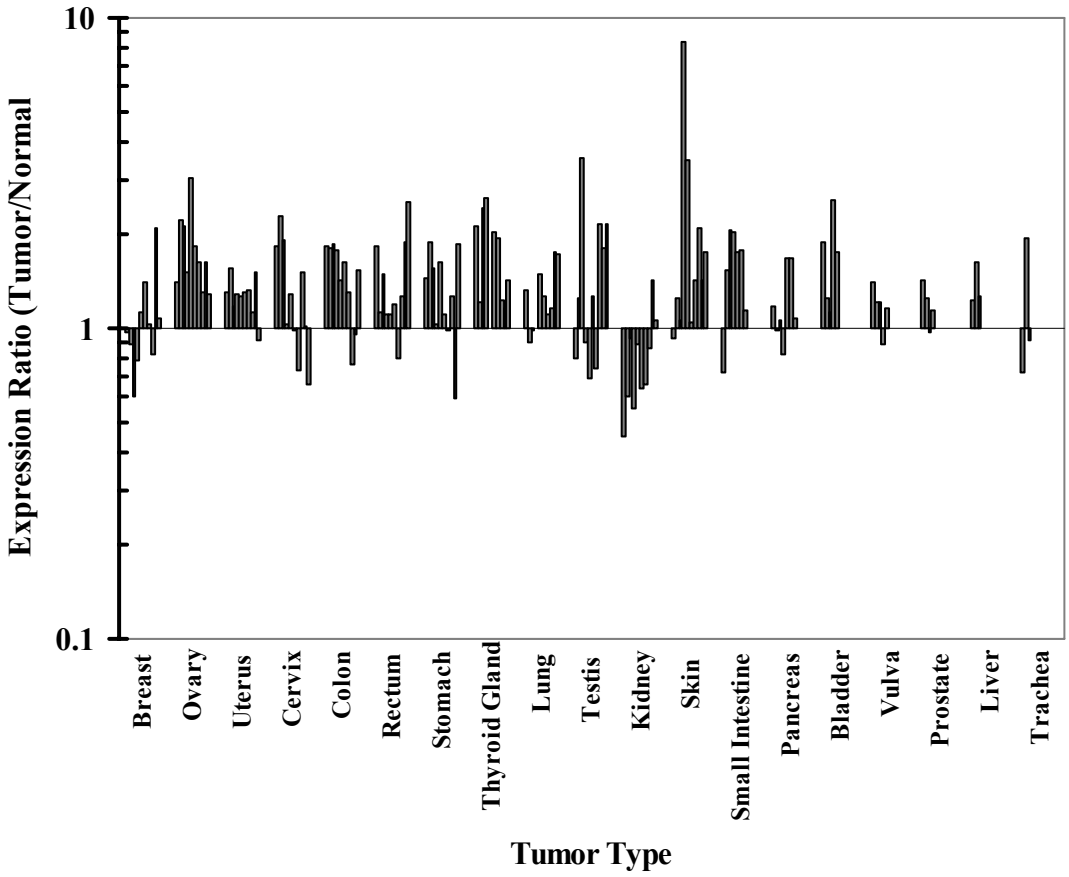
Tes
Ski
SmI
Pan
HeLa
Daudi
K562
HL60
G361
A549
MOLT4
SW480
Raji
Human Cancer
Cell Lines

Figure 23. Quantification of the hybridized Cancer Profiling Array.

ImageQuant was used to measure the signal intensity of each cDNA sample after both DUE-B and ubiquitin hybridizations. To measure the increase in DUE-B expression in tumor samples, the DUE-B hybridization signal was normalized to the ubiquitin signal in both the tumor and normal sample from each individual patient. Next the ratio of tumor sample to normal sample was taken to determine the fold increase in DUE-B expression in the specific patient's tumor. A ratio of less than one indicates a decrease in DUE-B expression in the individual tumor tissue.


10
1
0.1
Expression Ratio (Tumor/Normal
Breast
Ovary
Uterus
Cervix
Colon
Rectum
Stomach
Thyroid Gland
Lung
Testis
Kidney
Skin
Small Intestine
Pancreas
Bladder
Vulva
Prostate
Liver
Trachea
Tumor Type

Interestingly in kidney tumors DUE-B expression was overwhelmingly decreased in the analyzed samples (Figure 23). Further analysis of the blots in Figure 22 showed the presence of two cell lines (Daudi and Molt4) that appeared to lack any DUE-B mRNA expression. Daudi cells are a Burkitt's lymphoma cell line that harbors a translocation of one copy of the c-myc gene into the immunoglobulin heavy chain locus from a breakpoint more than 170 kb 5' to the c-myc core replicator (Joos et al, 1992). To confirm whether this lack of mRNA expression (Figure 24A) translated into a lack of protein expression, Daudi cells were obtained from ATCC and whole cell extracts prepared for western blot analysis. Although DUE-B protein from HeLa cells could be readily visualized, no signal could be detected in Daudi cell extract (Figure 24B) even when significantly higher total protein levels were analyzed (data not shown). Moreover treatment with azacytidine and trichostatin had no effect on DUE-B protein levels in Daudi cells (data not shown). These compounds are known to activate gene expression at the level of transcription by altering DNA methylation and histone acetylation status, respectively, at promoter elements. It is currently unclear how DUE-B expression is inhibited or regulated in Daudi cells. Interestingly these cells also contain the Epstein Barr virus, and recently it was shown that the EBV genome expresses five microRNAs that have significant complementarity to a number of important tumor suppressor genes (Pfeffer et al, 2004), including p53. Using the miRANDA algorithm (John et al, 2004) several potential matches of the EBV miRNAs to that of the DUE-B coding region and 5'- and 3'-untranslated regions were found (data not shown) suggesting that the lack of DUE-B expression in Daudi cells could be due to EBV, although this would clearly require further experimentation. Attempts to reintroduce DUE-B cDNAs into Daudi cells proved unsuccessful due to the difficulty in transfecting these cells.

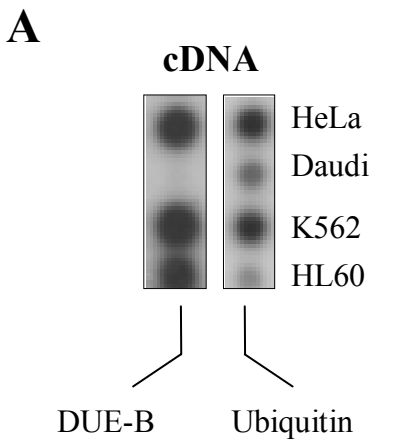
Summary

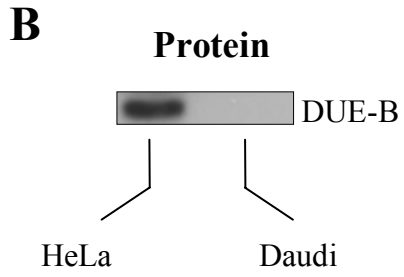
In summary, these data show DUE-B to be an abundant protein in human cells and *Xenopus* egg extracts that has the ability associate with chromatin and can be localized to replication origins *in vivo*. Furthermore DUE-B interacts with Dnmt1 and potentially other components of the chromosomal replication machinery, data that are consistent with a model whereby DUE-B is required for the formation or activation of replication complexes at origins of replication along chromosomes. Moreover, the overexpression of DUE-B in many tumor cells suggests a potential role as a marker for proliferation of

certain cell types. Together these results indicate a potential role for DUE-B in regulating the initiation of DNA replication at chromosomal replication origins.

Figure 24. The Burkitt's lymphoma cell line Daudi does not express DUE-B mRNA or protein.

(A) An area of the array from Figure 18 is enlarged to demonstrate a lack of detectable DUE-B expression in Daudi cells compared to other cancer cell lines. (B) Whole cell extracts (20 μg) prepared from HeLa cells and Daudi cells were run on SDS-PAGE and western blotted for DUE-B.


A
cDNA
HeLa
Daudi
K562
HL60
DUE-B
Ubiquitin


B
Protein
DUE-B
HeLa
Daudi

III. Functional characterization of DUE-B in human cells

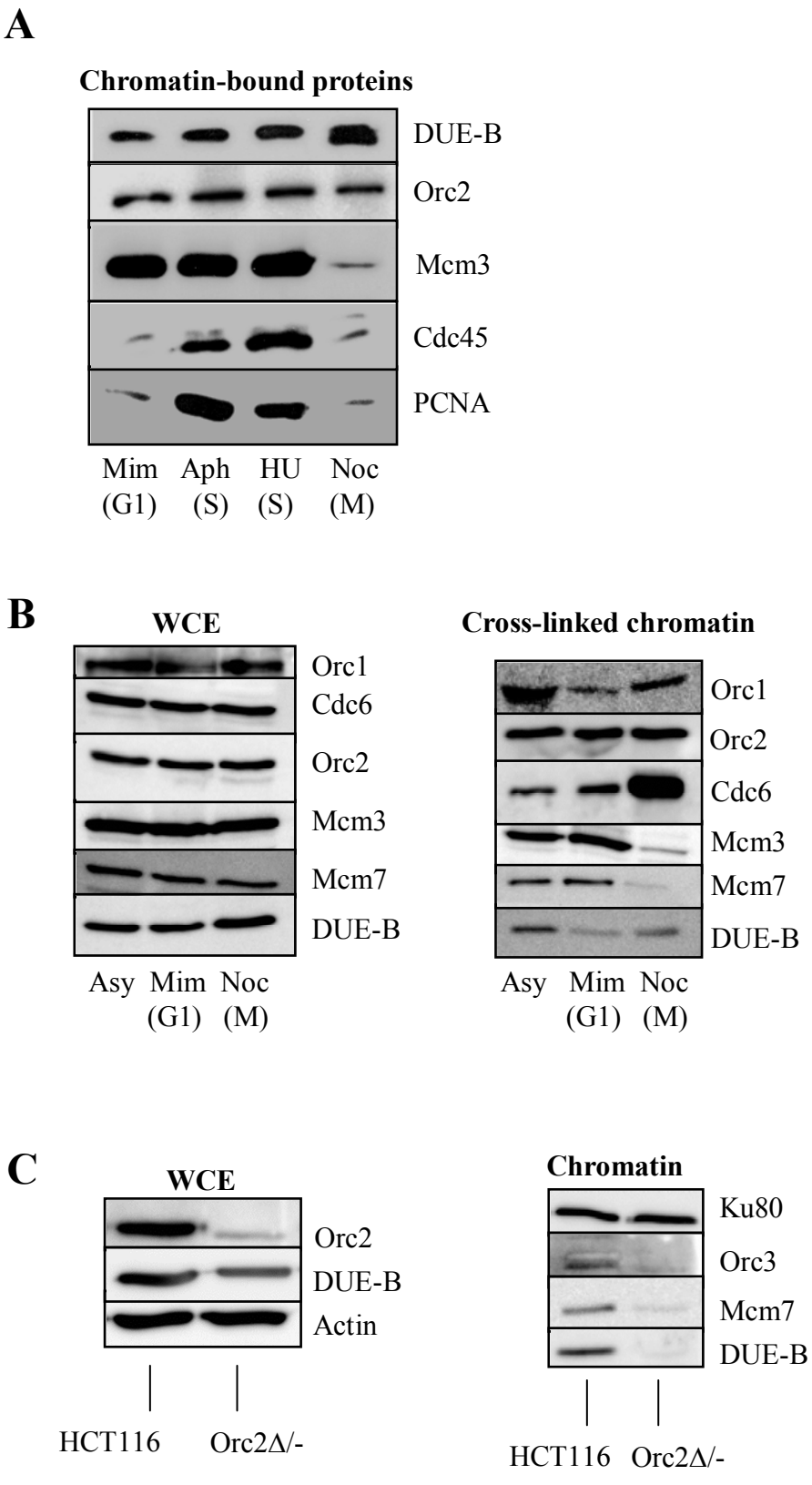
DUE-B binds chromatin throughout the cell cycle

To investigate the function of DUE-B in human cells we first investigated the binding of DUE-B to chromatin throughout the cell cycle, since many proteins involved in the formation of replication complexes bind replication origins, and hence chromatin, in a cell cycle-regulated fashion. As previous results had shown DUE-B protein expression to be stable throughout the cell cycle (Figure 16), we were curious whether its binding to chromatin might aid in a determination of its potential function. HeLa cells arrested at different phases of the cell cycle were fractionated to enrich for chromatin-bound proteins. The fractionation scheme separates proteins known to be exclusively cytoplasmic from those that are entirely bound to chromatin (Figure 17B). The chromatin fractions from the different cell cycle phases were then analyzed by SDS-PAGE and western blotting to look at several replication proteins involved in pre-RC formation, activation, or replication elongation. As previously observed, Orc2 bound to replication origins throughout the cell cycle (Mendez and Stillman, 2000), and the fractionation of HeLa cells recapitulated this observation (Figure 25A). In contrast to that of Orc2, Mcm3 was found only on chromatin from G1 and S phase cells, consistent with its binding to replication origins in G1 during the formation of pre-RCs and its role as the putative replicative helicase during S phase. MCMs are reported to come off the chromatin during S phase as replication completes (Mendez and Stillman, 2000), and thus were absent from mitotic chromatin. Cdc45, a co-factor for the MCM helicase was found only on chromatin from S phase-arrested cells, similar to the behavior of PCNA, a DNA polymerase processivity factor. The pattern of DUE-B binding to chromatin was most closely related to that of Orc2 in that it was bound to chromatin throughout the cell cycle. Interestingly, its binding may increase during S phase as it appeared to be elevated on mitotic chromatin.

To confirm that DUE-B is bound to chromatin throughout the cell cycle, a separate chromatin enrichment procedure was employed that used formaldehyde crosslinking to covalently bind proteins to DNA. This protocol was also used in conjunction with immunoprecipitation to determine the distribution of replication proteins across the c-myc replicator (Ghosh et al, 2006). Crosslinked chromatin was prepared from asynchronous cells, as well as from cells arrested in late G1 with mimosine or in mitosis with nocodazole. The data are presented in Figure 25B, and again showed slightly higher levels of DUE-B

Figure 25. DUE-B binds chromatin throughout the cell cycle in an Orc2-dependent manner.

(A) DUE-B is bound to chromatin throughout the cell cycle, but at slightly elevated levels on mitotic chromatin. HeLa cells were fractionated as described previously from cells arrested at different phases of the cell cycle with the indicated drugs. Fractions corresponding to an equivalent number of cells were analyzed by SDS-PAGE and western blotting with antibodies against the indicated proteins Comparison of DUE-B levels on mitotic chromatin to that on G1 chromatin suggests a higher level of binding. (B) Using a separate fractionation method, in which HeLa nuclei are cross-linked with formaldehyde to stabilize protein binding on DNA, DUE-B is also found to be bound to chromatin but at a slightly higher level in mitosis. Note that ORC1, CDC6, and DUE-B all display elevated binding to mitotic chromatin compared to chromatin from cells arrested with mimosine. The expression level of all the replication proteins in whole cell extracts (WCE) is also shown (left) to demonstrate that decreased binding to chromatin is not due to an absence of protein expression. (C) HCT116 cells and a hypomorphic cell line expressing a low amount of a truncated form of Orc2 (Orc2Δ/-) were fractionated to enrich for chromatin-bound proteins. Whole cell extracts (WCE) and chromatin fractions from equivalent numbers of cells were analyzed by SDS-PAGE and western blotting.


A
Chromatin-bound proteins
DUE-B
Orc2
Mcm3
Cdc45
PCNA
Mim (G1)
Aph (S)
HU (S)
Noc (M)
B
WCE
Orc1
Cdc6
Orc2
Mcm3
Mcm7
DUE-B
Asy
Mim (G1)
Noc (M)
Cross-linked chromatin
Orc1
Orc2
Cdc6
Mcm3
Mcm7
DUE-B
Asy
Mim (G1)
Noc (M)
C
WCE
Orc2
DUE-B
Actin
HCT116
Orc2Δ/-
Chromatin
Ku80
Orc3
Mcm7
DUE-B
HCT116
Orc2Δ/-

on mitotic chromatin compared to that found on chromatin from cells arrested in late G1. Interestingly, this same pattern was observed for both Orc1 and Cdc6, both of which are reported to interact only transiently with replication origins and may function in origin selection. Cdc6 was reported to be involved in regulating the stability of ORC on chromatin (Harvey and Newport, 2003), and thus may serve in some capacity with Orc1 to determine where along chromosomes to load MCMs and build pre-RCs. The elevated level of Cdc6 on mitotic chromatin was consistent with the start of pre-RC formation and origin selection that occurs in the later stages of mitosis and early G1 phase. That the pattern of binding is similar to that of DUE-B may suggest that DUE-B cooperates with these proteins to select sites along chromosomes on which to build the replication machinery.

Since the assembly of replication complexes at origins occurs in stepwise fashion, it was of interest to determine whether the binding of DUE-B to chromatin depends on prior binding of other replication initiation proteins. ChIP analyses of mutant c-myc replicator constructs demonstrated that DUE-B and Mcm3 bind to the c-myc replicator independently (Ghosh et al, 2006). Thus we were interested in whether DUE-B binding to chromatin required the prior binding of the ORC. HCT116 cells and an HCT116 cell line hypomorphic for Orc2 (Orc2Δ/-) were utilized for biochemical fractionation to enrich for chromatin-bound proteins, and then analyzed by SDS-PAGE and western blotting. As previously reported (Dhar et al, 2001) and indicated in Figure 25C, the Orc2Δ/- cells expressed significantly less Orc2 than the parental HCT116 cells. The remaining low level of protein that was expressed is an N-terminally truncated Orc2 protein. These cells show a prolonged G1 phase of the cell cycle, but are still able to initiate DNA synthesis at the c-myc and β-globin replication origins and continue to show similar replication fork densities as that found in wild-type HCT116 cells (Dhar et al, 2001; Teer et al, 2006). It was recently shown that these cells show defects in pre-RC assembly, with less Mcm7 on chromatin and at the lamin B2 replication origin (Teer et al, 2006). To determine if the binding of DUE-B to chromatin is affected in the Orc2Δ/- cells, an equal number of HCT116 and Orc2Δ/- cells were fractionated to enrich for chromatin bound proteins. As previously reported, pre-RC assembly was inhibited, with reduced levels of Orc3 and Mcm7 on chromatin in Orc2Δ/- cells (Figure 25C). Interestingly very little DUE-B could be observed on the chromatin in these cells (Figure 25C), suggesting that DUE-B recruitment to chromatin, and thus to replication origins, is dependent upon Orc2 or the entire origin recognition complex (ORC1-6).

RNAi can be used to knock-down DUE-B levels in human cells

To study the function of DUE-B at replication origins and on chromatin, short interfering RNA (siRNA) technology was employed to reduce cellular levels of DUE-B and investigate phenotypic changes that occur after depletion of the protein. Two independent siRNAs were designed that target different regions of the DUE-B mRNA for degradation via the RNAi pathway. A control, predicted non-targeting siRNA that contained the same nucleotide content of DUE-B siRNA#1 but in a scrambled order was used as a negative control siRNA throughout these experiments. An siRNA targeting Ku80 was used in several experiments as an alternative control for RNAi. The target sequence for each of the siRNAs is provided in Table 4.

HeLa cells plated at low density were transfected with the indicated siRNAs either once or twice over a period of 4 days, and were then harvested for the preparation of whole cell extracts. As indicated in Figure 26A, both DUE-B siRNA#1 and siRNA#2 efficiently reduced DUE-B protein levels within 48 hours after a single transfection, but did not negatively affect the levels of Ku80 protein. Similarly, knock-down of Ku80 had no effect on DUE-B protein levels. Although both DUE-B siRNA#1 and siRNA#2 were effective in reducing DUE-B protein levels, initial experiments had indicated that DUE-B siRNA#2 could do so more effectively in a shorter period of time. To confirm this observation and use a non-targeting siRNA as a negative control, HeLa cells were transfected with the indicated siRNAs either once or twice over 4 days, then harvested for western blot analysis. Again, DUE-B siRNA#2 was able to reduce cellular levels of DUE-B more efficiently than DUE-B siRNA#1 (Figure 26B). Whereas DUE-B siRNA#1 would typically reduce DUE-B protein levels by 80-90% within 48 hours after a single transfection, DUE-B siRNA#2 could reduce levels by >95% under the same conditions.

To examine whether the siRNAs could function to reduce DUE-B protein levels in other human cancer cell lines, HCT116 cells were transfected with siRNAs twice over a period of 4 days and whole cell extracts analyzed by western blotting. Again DUE-B siRNA#2 reduced DUE-B protein levels slightly better than DUE-B siRNA#1. Neither the control siRNA nor the Ku80 siRNA had any significant effect on DUE-B protein levels.

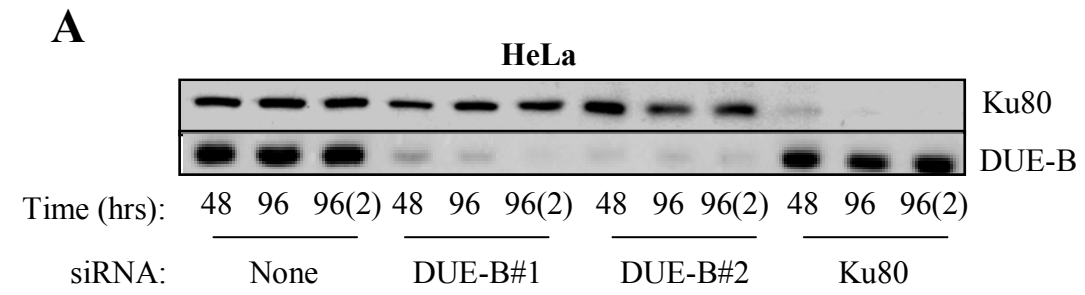
Table 4. List of siRNAs used in these studies.

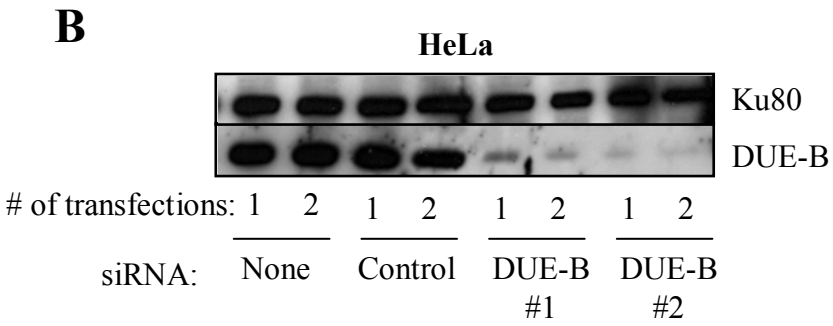
Small, interfering RNAs (siRNAs) were designed against to target the indicated sequences and were obtained pre-annealed from Xeragon (Qiagen) and Dharmacon. The siRNAs are each 21bp long with dTdT dinucleotides at the 3'-termini. The control siRNA is predicted not to target any known human mRNA, based on Blast searches of the NCBI and human genome databases. The target location of the indicated siRNAs is provided.

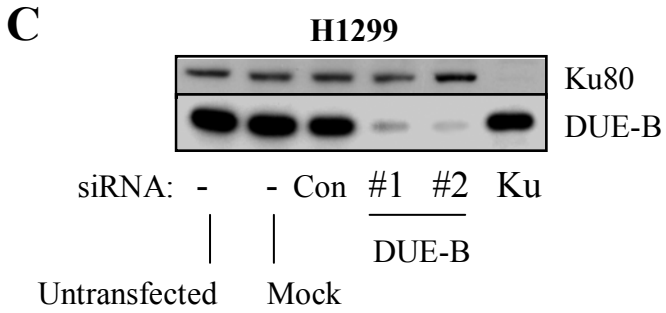
siRNA	Target Sequence	Starting Location	Gene
Control	AAGAGAGAAAGTGTCTCTC	-	Non-targeting
DUE-B #1	AAACAGTACGAGATTCTGTGT	209	DUE-B cDNA
DUE-B #2	AAGCACTGGTCGAAGAGTGTG	182	DUE-B cDNA
Ku80	AACGAAAATCCAACCAGGTTC	287	Ku-86 ORF NM021141

Figure 26. siRNAs can be used to reduce DUE-B protein expression in human cells.

(A) HeLa cells plated at low density into 24-well plates were transfected once with the indicated siRNAs (80 nM) using Oligofectamine reagent at time 0 or transfected against after 48 hours. Cells were harvested after 48 or 96 hours as indicated, lysed with M-PER buffer containing protease inhibitors, run on SDS-PAGE, and analyzed by western blotting for DUE-B and Ku80. Cells exposed to transfection reagent but no siRNA are indicated in lanes 1-3. Cells transfected twice with siRNA are indicated as "96(2)". (B) To confirm the apparent difference between the two siRNAs in the effectiveness of reducing DUE-B protein levels, HeLa cells were transfected using Oligofectamine reagent twice (at time 0 and 48 hours) with the indicated siRNAs and harvested at 96 hours for western blot analysis. In this experiment a non-targeting siRNA was included as a control. (C) DUE-B expression in HCT116 cells can also be reduced using RNAi. HCT116 cells were transfected twice with the indicated siRNAs (Con, control siRNA) and harvested for protein analysis at 96 hours.


A
HeLa
Ku80
DUE-B
Time (hrs): 48 96 96(2) 48 96 96(2) 48 96 96(2) 48 96 96(2)
siRNA: None DUE-B#1 DUE-B#2 Ku80


B
HeLa
Ku80
DUE-B
# of transfections: 1 2 1 2 1 2 1 2
siRNA: None Control DUE-B #1 DUE-B #2


C
H1299
Ku80
DUE-B
siRNA: - - Con #1 #2 Ku
DUE-B
Untransfected Mock

DUE-B RNAi inhibits cell proliferation and induces cell death

To examine the phenotypic effects the DUE-B siRNAs, HeLa cells were transfected twice over 4 days either in the absence of siRNA or with a control siRNA or the DUE-B siRNA. Cells depleted of DUE-B protein via RNAi showed a significant reduction in total cell number after 4 days indicating that knocking down DUE-B inhibits cell proliferation (Figure 27A). To examine whether the apparent inhibition of proliferation was due to induction of cell death, cells were stained with trypan blue after 4 days of growth and two transfections with the indicated siRNAs. As indicated in Figure 27B, a significant increase in the number of trypan blue-positive cells was observed, indicating that reduction of DUE-B protein levels caused cells to die. In both the cell counting assay and trypan blue staining assay, DUE-B siRNA#2 had a more pronounced affect than DUE-B siRNA#1, consistent with the more efficient ability to reduce DUE- B protein levels.

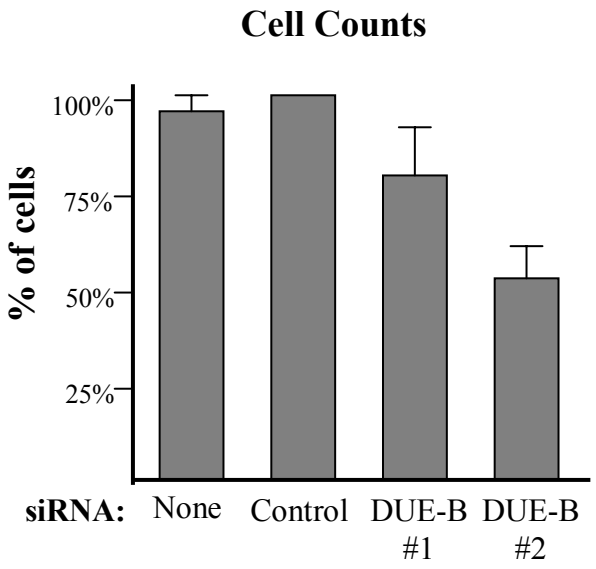
In an attempt to determine if the cell proliferation defect was due to cells becoming arrested in a particular phase of the cell cycle, cells transfected twice with DUE-B siRNA#2 were harvested after 96 hours and analyzed for DNA content by flow cytometry. As demonstrated in Figure 28A the most apparent change in cell cycle profile was the emergence of a population of cells with a sub-G1 phase DNA content. These cells typically represent either apoptotic or dead cells as a result of DNA fragmentation and degradation. The presence of this sub-G1 population of cells was consistent with trypan blue staining indicating a significant (4- to 6-fold) increase in the number of dead or dying cells after transfection with DUE-B siRNA#2 (Figure 28B). Similar to the results presented in Figure 27, a sub-G1 population was observed after transfection with DUE-B siRNA#1, but was found to be smaller than that observed with DUE-B siRNA#1. Again we have attributed this result to the difference in potency of the two DUE-B siRNAs. Although we observed a large increase in the number of sub-G1 cells after DUE-B knock-down, we were usually unable to determine from asynchronous populations of cells whether there was any defect in progression through a specific phase of the cell cycle.

Since it had been suggested that siRNAs may potentially activate the p53 tumor suppressor protein non-specifically (Scacheri et al, 2004), and p53 is known to be a potent regulator of cell cycle progression

Figure 27. DUE-B RNAi inhibits cell proliferation and induces cell death in HeLa cells.

(A) HeLa cells were transfected with 80 nM of the indicated siRNAs at time 0 and again at 48 hours. After 96 hours (from the initial transfection), cells were trypsinized and counted on a hemacytometer slide. The data represent the average (and standard deviation) from three independent experiments, and are normalized to the total cell number from the cells transfected with control siRNA. (B) HeLa cells were transfected with siRNAs as described above, but instead stained with trypan blue to determine the number of dead cells that had accumulated after two siRNA transfections and 4 days in culture. The graph shows data from a representative experiment.

A


Cell Counts
100%
75%
50%
25%
% of cells
siRNA:
None
Control
DUE-B #1
DUE-B #2

B

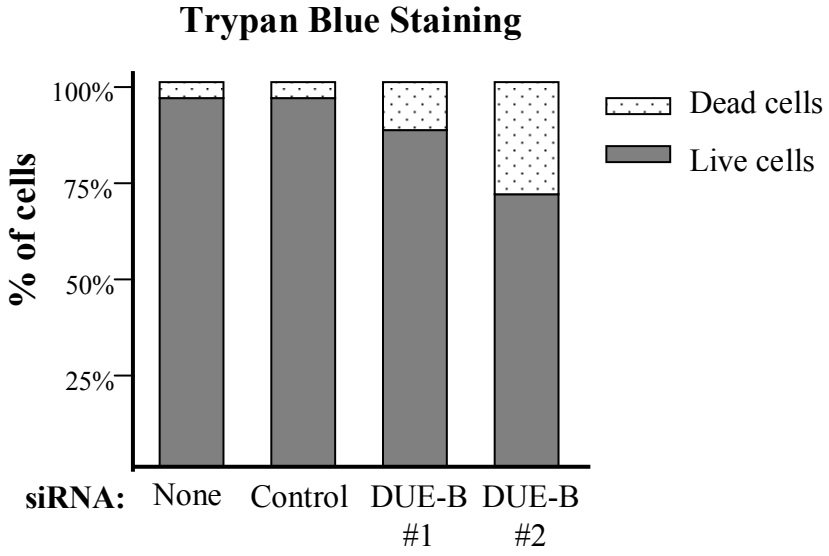
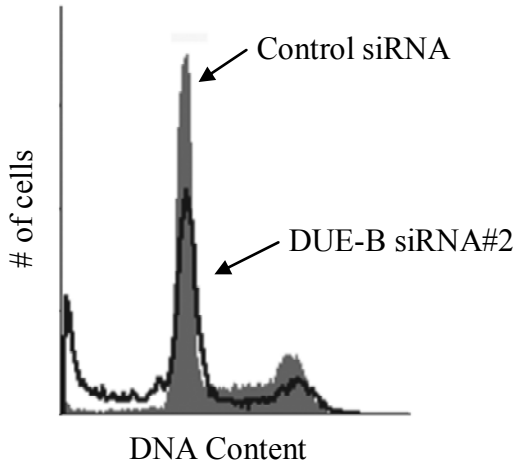
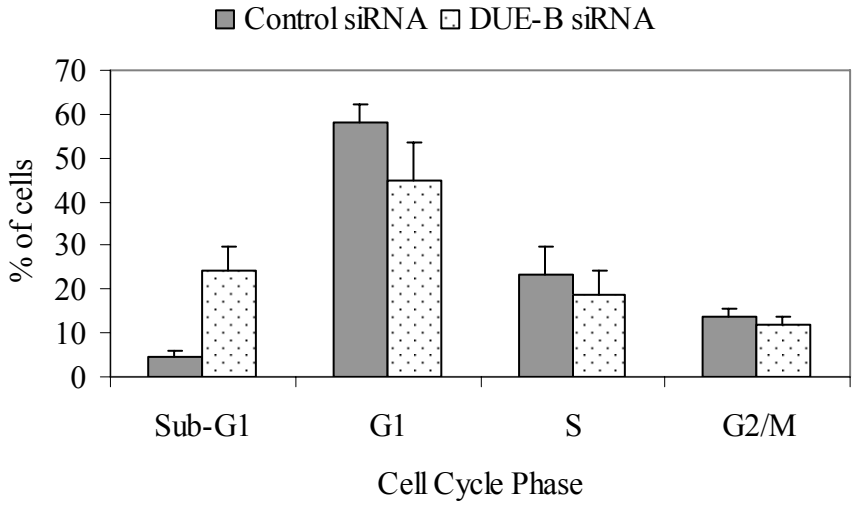

Trypan Blue Staining
100%
75%
50%
25%
% of cells
siRNA:
None
Control
DUE-B #1
DUE-B #2
Dead cells
Live cells

Figure 28. Flow cytometric analysis of DNA content indicates that DUE-B RNAi induces cell death. (A) Propidium iodide-stained HeLa cells transfected with either control or DUE-B siRNA#2 as described in Figure 36 were analyzed by flow cytometry to monitor cell cycle profile and to determine the number of dead or dying cells (sub-G1 population). The flow profile shows data from a representative experiment. (B) Data from four independent experiments analyzed by flow cytometry as above were compiled, and the percentage of cells in each phase of the cell cycle after transfection with either control or DUE-B siRNA were averaged (with standard deviation). Similar results were obtained in cells transfected with DUE-B siRNA#1, but with a lower subG1 population of cells compared to cells transfected with DUE-B siRNA#2.

A


Control siRNA
DUE-B siRNA#2
# of cells
DNA Content

B


Control siRNA
DUE-B siRNA
% of cells
70
60
50
40
30
20
10
0
Sub-G1
G1
S
G2/M
Cell Cycle Phase

due to its downstream effects on transcription, we were interested in whether the effect of DUE-B siRNA was due to activation of the p53 pathway. To investigate this hypothesis, H1299 (p53-null), HCT116 (wild-type p53), and HeLa (p53-inactivated) cells were used alongside one another in transfection experiments with either control or DUE-B siRNA#2. As previously demonstrated (Figure 26C), DUE-B siRNA#2 efficiently and specifically lowered DUE-B protein levels in all three human cancer cell lines (Figure 29A). HeLa cells, which contain wild-type p53 that is inactivated due to the human papillomavirus E6 protein, showed no detectable stabilization of p53 protein levels. In HCT116 cells, which contain wild-type p53, we found that protein levels were somewhat destabilized after DUE-B knockdown, and the significance of this result is currently unclear. Regardless, in all three cell lines DUE-B knockdown resulted in decreased proliferation (Figure 29B) and increased trypan blue staining (Figure 29C). The difference in the magnitude of the effects on cell proliferation and cell death are presumably due to differences in cell physiology, but are independent of the p53 status of the cell type.

DUE-B RNAi inhibits the G1/S transition

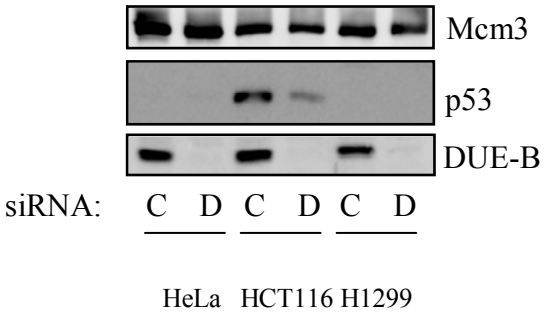
To better determine whether the defect in cell proliferation was due to cell cycle checkpoint activation or an inability to efficiently move through a particular phase of the cell cycle, HeLa cells transfected with DUE-B siRNA#2 were blocked in either mitosis with nocodazole (Figure 30A) or in late G1 phase with mimosine (Figure 30B). Cells were then released from the drug-induced cell cycle arrest and allowed to progress through the cell cycle until a significant number of control siRNA-transfected cells had entered S phase, as determined by DNA content analysis using flow cytometry. Regardless of the treatment employed, it was apparent that cells transfected with DUE-B siRNA became arrested with fewer cells with an S phase DNA content and more cells with a G1 phase DNA content (Figure 30). Depending on the timing of the cell cycle block and release, and on the extent of RNAi-mediated DUE-B depletion, the cells arrested in G1 eventually began to replicate DNA and enter S phase. These results are consistent with a proposed role of DUE-B at replication origins where DUE-B may be required for the initiation of DNA synthesis.

To confirm that the effect of DUE-B knock-down on cell cycle arrest was not unique to DUE-B siRNA#2, HeLa cells were transfected with either DUE-B siRNA#1 or DUE-B siRNA#2 to reduce levels of DUE-B protein (Figure 31A) and then released from a late G1 phase cell cycle block mediated by

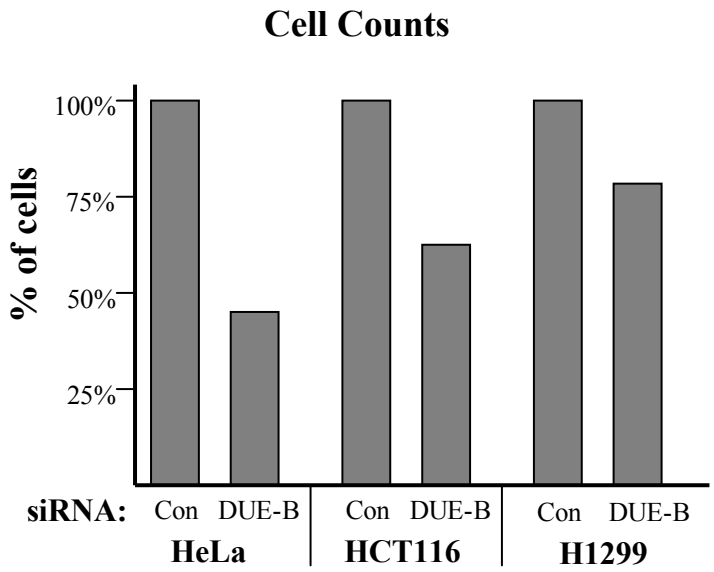
Figure 29. DUE-B RNAi inhibits cell proliferation and induces cell death in a p53-independent manner.

HeLa, HCT116, and H1299 cells were transfected with either a control siRNA or an siRNA targeting DUE-B twice over 4 days (Time 0 and 48 hours). A portion of the cells were taken for the preparation of whole cell extracts, and where then analyzed by western blotting against the indicated proteins (A). The remaining cells were counted on a hemocytometer to determine the cell population size (B) or else stained with trypan blue to measure the percentage of dead and/or dying cells (C).

A


Mcm3
p53
DUE-B
siRNA: C D C D C D
HeLa HCT116 H1299

B


Cell Counts
% of cells
100%
75%
50%
25%
siRNA: Con DUE-B Con DUE-B Con DUE-B
HeLa
HCT116
H1299

C

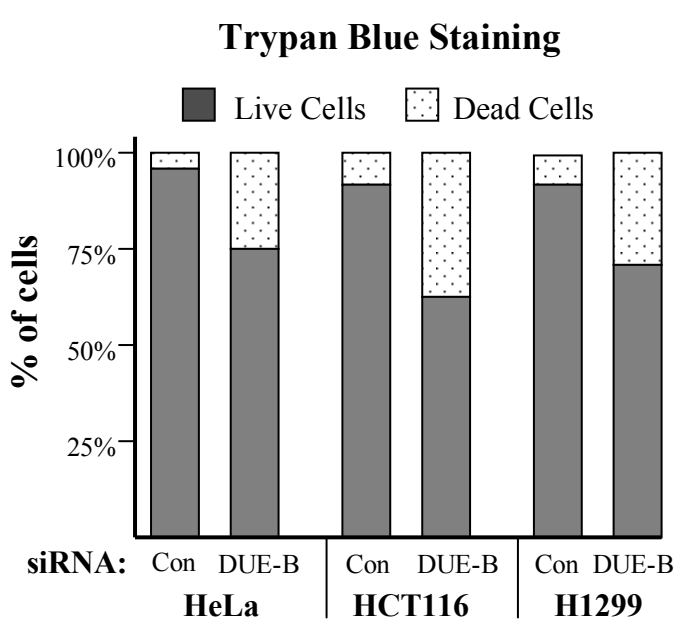

Trypan Blue Staining
Live Cells
Dead Cells
% of cells
100%
75%
50%
25%
siRNA: Con DUE-B Con DUE-B Con DUE-B
HeLa
HCT116
H1299

Figure 30. DUE-B RNAi inhibits entry of cells into S phase.

HeLa cells transfected with either control siRNA or DUE-B siRNA#2 were arrested in either mitosis by treatment with nocodazole (A) or in late G1 phase by treatment with mimosine (B). Cells were then released into fresh medium to allow cells to progress through the cell cycle and move into S phase. Cells were then harvested and fixed, stained with propidium iodide, and analyzed for DNA content using flow cytometry. Synchronizations with the indicated drugs typically lasted from 18-24 hours to arrest cells in mitosis or late G1. Movement into S phase was typically detected after ~14-15 hours after the arrest in mitosis with nocodazole. Release of cells from the late G1 block with mimosine into S phase was observed within 6 to 9 hours after addition of drug-free medium.

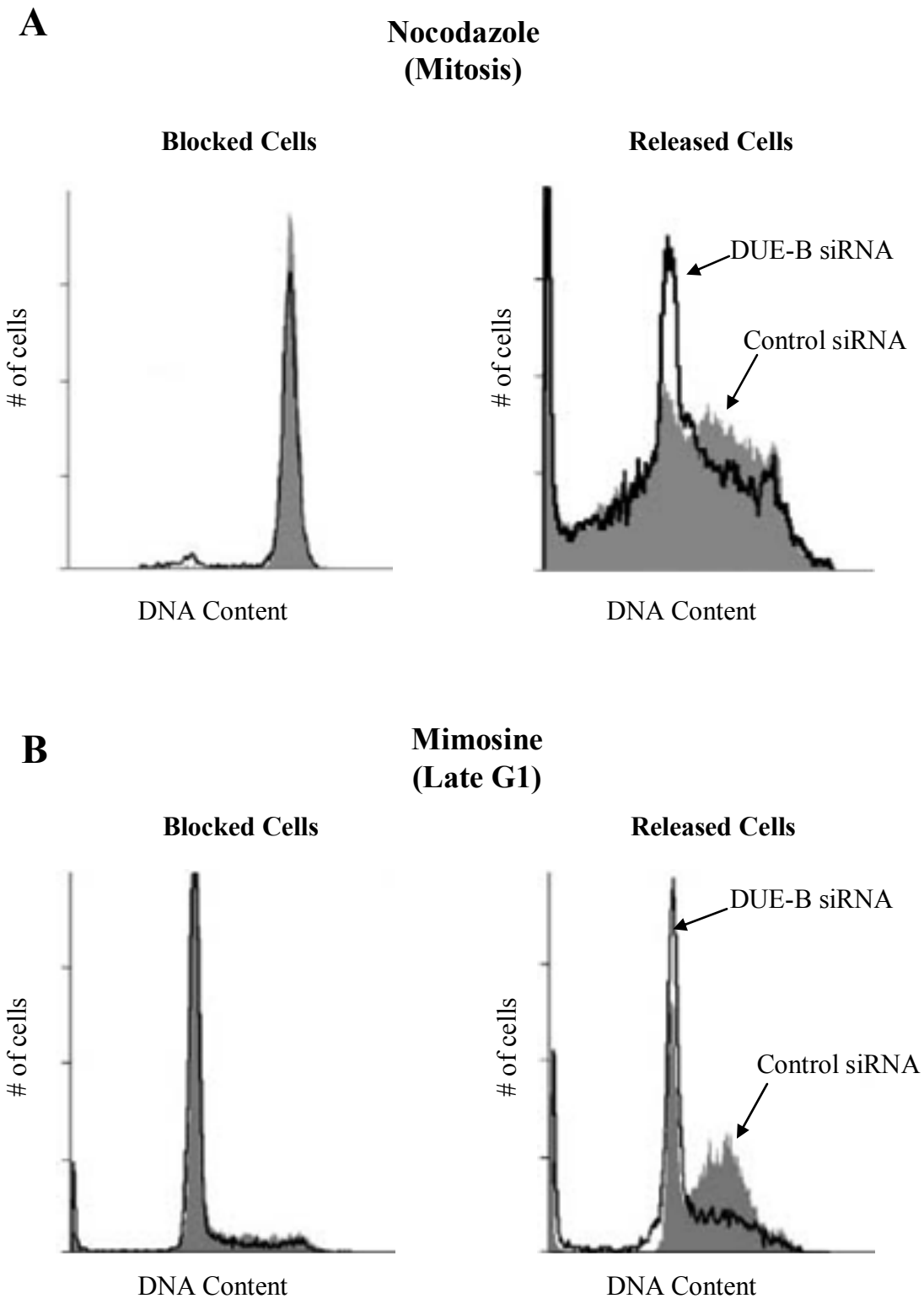
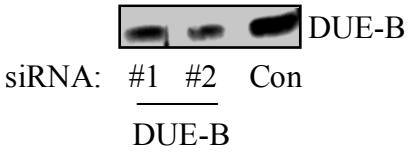

A
Nocodazole
(Mitosis)
Blocked Cells
Released Cells
# of cells
DUE-B siRNA
Control siRNA
DNA Content
DNA Content
B
Mimosine
(Late G1)
Blocked Cells
Released Cells
# of cells
DUE-B siRNA
Control siRNA
DNA Content
DNA Content

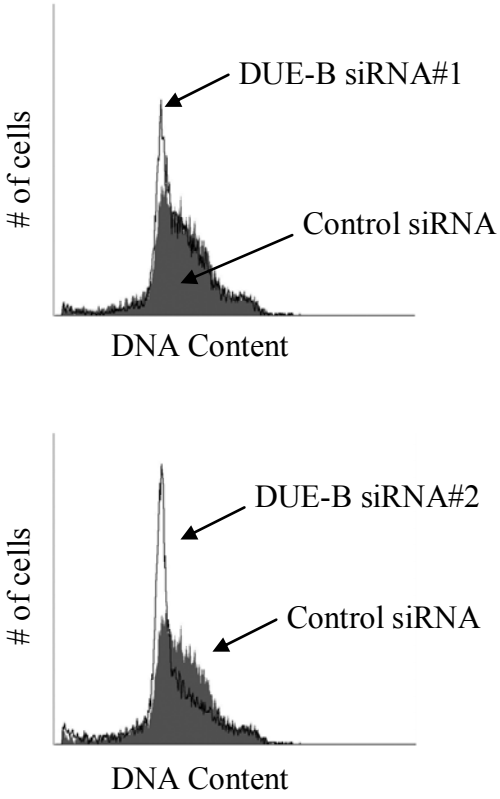
Figure 31. Multiple DUE-B siRNAs block cell cycle progression at the G1/S boundary.

HeLa cells were transfected twice with 80 nM DUE-B siRNA#1, DUE-B siRNA#2, or a control siRNA using Oligofectamine transfection reagent. Immediately after the second transfection, cells were treated with 500 μM mimosine for 20 hours. Cells were released from the late G1 arrest for 6 hours, then harvested for protein analysis by western blotting (A), for DNA content by flow cytometry (B). The distribution of cells at various stages of the cell cycle after release from the mimosine block is indicated (C).

A



B



C

siRNA	SubG1	G1	S	G2/M
Control	5%	35%	57%	3%
DUE-B#1	3%	44%	51%	2%
DUE-B#2	7%	55%	36%	2%

mimosine treatment. Analysis by flow cytometry suggested that a G1 arrest was induced with both DUE-B siRNA#1 and siRNA#2, but not the control siRNA (Figure 31B). Analysis of the flow cytometry profiles confirmed a G1 arrest with both DUE-B siRNAs, but indicated that DUE-B siRNA#2 was more effective in inducing this phenotype (Figure 31C). Again this has been attributed to the difference in efficiency of DUE-B knockdown between the two DUE-B siRNA constructs. Attempts to look at cell cycle progression after release from a mitotic block showed no apparent difference between the control siRNA and DUE-B siRNA#1. Since nocodazole arrests cells much earlier in the cycle than mimosine, it is likely the additional time in G1 coupled with the less efficient knockdown by DUE-B siRNA#1 allows cells enough time to overcome the defect that DUE-B depletion causes.

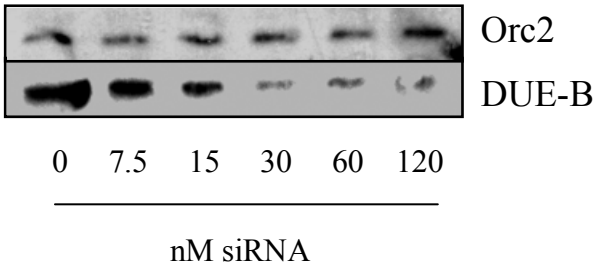
To test whether there is a correlation between the extent of DUE-B depletion and the inhibition of entry into S phase, HeLa cells were transfected with control or DUE-B siRNA#2 twice over 24 hours before arresting in late G1 phase with mimosine. Cells were then released for 7 hours into S phase and harvested for western blot analysis to detect DUE-B protein levels and for DNA content analysis by flow cytometry to determine defects in DNA synthesis. The effect of DUE-B siRNA on DUE-B protein levels was found to be dose dependent up to around 30 nM to 60 nM siRNA, after which protein levels were not significantly reduced any further (Figure 32A). Interestingly the same observation was made when cells were analyzed by flow cytometry (Figure 32B), with DUE-B siRNA between 30 nM and 120 nM yielding a similar percent of cells remaining with a G1 phase DNA content (Figure 32C). The correlation between the extent of DUE-B depletion and the S phase entry defect suggest the effect of DUE-B siRNA is not likely due to non-specific effects on cell cycle or on other mRNA targets.

To show that the effect of DUE-B RNAi on S phase entry is not unique to HeLa cells, H1299 cells were transfected once with 40 nM DUE-B siRNA#2 or a control siRNA and allowed to remain asynchronous or else arrested in late G1 with mimosine and then allowed to recover from the cell cycle arrest and synthesize DNA. Even in asynchronous H1299 cells it was apparent that there was a small increase in the percentage of cells remaining in G1 after DUE-B knockdown (Figure 33A and B). H1299 cells depleted of DUE-B also showed fewer cells in S phase after release from a mimosine block (Figure 33C), further suggesting a role for DUE-B at the G1 to S phase transition of the cell cycle in human cells.

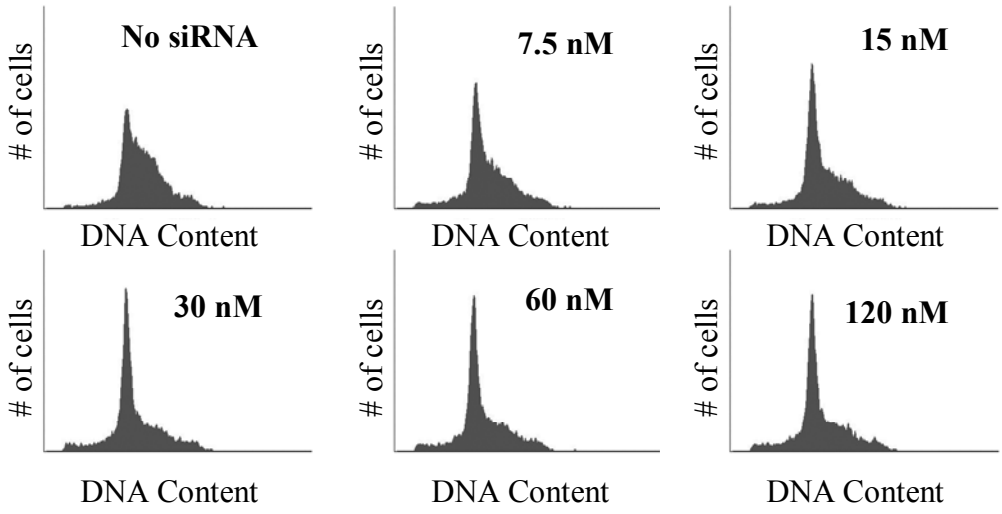
Figure 32. Effect of DUE-B siRNA on DUE-B protein expression level and on entry of cells into S phase is dose-dependent.

HeLa cells at ~30% confluence were transfected using Oligofectamine reagent with the indicated concentration of DUE-B siRNA#2 twice (at time 0 and again after 24 hours), then treated with 500 μM mimosine for 20 hours. Cells were then released for 7 hours from the late G1 block, and cells were harvested for protein analysis by western blotting (A) or for DNA content by flow cytometry (B). The relative number of cells in each phase of the cell cycle under each transfection condition is indicated (C).

A



B

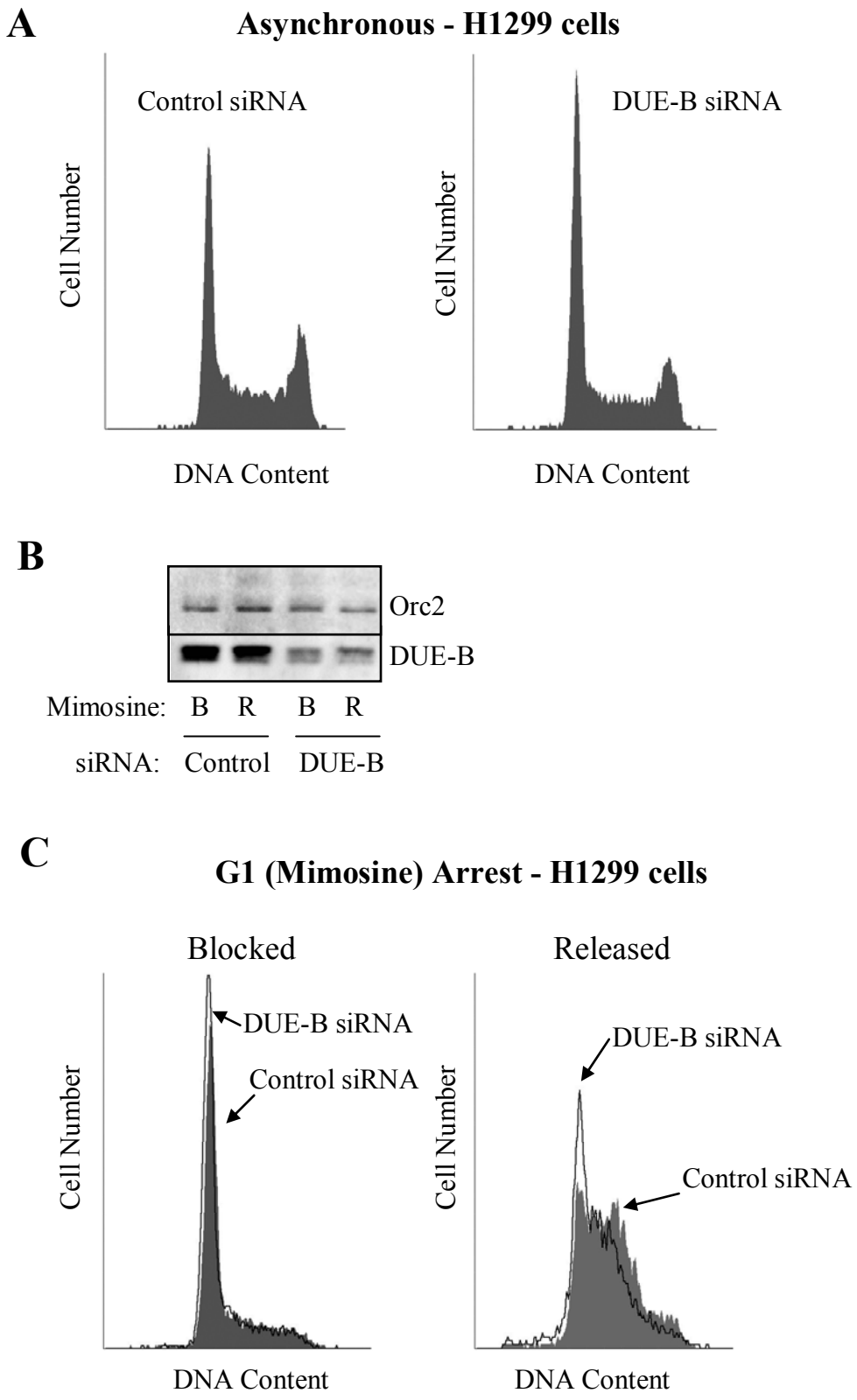


C

[siRNA]	SubG1	G1	S	G2/M
0	3%	31%	63%	3%
7.5 nM	7%	41%	49%	3%
15 nM	6%	45%	46%	3%
30 nM	9%	48%	40%	3%
60 nM	9%	51%	38%	2%
120 nM	10%	49%	39%	2%

Figure 33. DUE-B RNAi inhibits entry into S phase in H1299 cells.

(A) H1299 cells transfected at ~30% confluence once with 40 nM siRNA (control or DUE-B) using Lipofectamine 2000 were grown for 36 hours. Cells were then fixed, stained with propidium iodide, and analyzed by flow cytometry. (B) Six hours after transfection with siRNAs (as described above), 500 μM mimosine was added and the cells were allowed to arrest in late G1 phase for 20 hours (B, blocked cells). Fresh medium was added to release the cells into S phase (R, released cells), and 6.5 hours later the cells were harvested for analysis by western blotting (B) and flow cytometry (C). Transfections were performed in duplicate wells of a 6-well plate, with 1 well used for protein analysis and one for flow cytometry (B, blocked cells; R, released cells).


A
Asynchronous - H1299 cells
Control siRNA
DUE-B siRNA
Cell Number
Cell Number
DNA Content
DNA Content
B
Orc2
DUE-B
Mimosine: B R B R
siRNA: Control DUE-B
C
G1 (Mimosine) Arrest - H1299 cells
Blocked
Released
DUE-B siRNA
Control siRNA
DUE-B siRNA
Control siRNA
Cell Number
Cell Number
DNA Content
DNA Content

Cells can also be arrested in early S phase by prolonged treatment with either aphidicolin or hydroxyurea. Aphidicolin inhibits the replicative DNA polymerases and hydroxyurea, through inhibition of ribonucleotide reductase, depletes nucleotides required for DNA replication. As demonstrated earlier, cells arrested with mimosine lack chromatin-bound Cdc45, whereas cells arrested with either aphidicolin or hydroxyurea contain Cdc45 on their chromatin (Figure 25A), consistent with evidence that the latter drugs affect S phase events occurring after pre-RC activation. To compare whether a difference in phenotype could be observed in DUE-B depleted cells depending on whether the cells were arrested in late G1 or early S phase, HeLa cells transfected with control or DUE-B siRNA were arrested with either mimosine or aphidicolin, then allowed to move through S phase. Consistent with data already presented, cells depleted of DUE-B show a defect in S phase entry after release from the late G1 arrest caused by mimosine. That all the cells are able to enter S phase and move into mitosis argue that the effect of DUE-B depletion is not an irreversible G1 arrest. Similarly, DUE-B depleted cells could be effectively blocked in early S phase with aphidicolin, but interestingly these cells do not show any further defect in DNA synthesis as the cells are able to move through S phase with normal kinetics (Figure 34). Similar results were obtained when hydroxyurea was used to synchronize cells in early S phase, since DUE-B depleted cells moved equally as far into S phase as control siRNA-transfected cells after release from the hydroxyurea block (Figure 35). These data argue for a requirement for DUE-B at a step between where mimosine and aphidicolin or hydroxyurea arrest the cell cycle and suggest that DUE-B plays a role at the replication origin at step between establishment of pre-RCs (G1 phase) and the formation of active replication forks (S phase).

DUE-B siRNA alters replication protein binding to chromatin

Since the binding of specific replication proteins to replication origins can be used to define the state of the replication origin for DNA synthesis, we were interested in whether DUE-B depletion was correlated with the loss of particular replication proteins on chromatin or at replication origins. ChIP data from our laboratory demonstrated that the removal of DNA sequences containing a DUE from the c-myc replicator abolished DUE-B but not Orc2 or Mcm3 binding to the replicator. We were thus interested in whether a similar observation could be made genome-wide by examining the binding of these proteins to bulk chromatin. HeLa cells depleted of DUE-B via RNAi were fractionated to enrich for chromatin-bound proteins. Similar to that observed for the c-myc replicator, loss of DUE-B on HeLa cell chromatin had no

Figure 34. DUE-B siRNA inhibits DNA synthesis at the G1/S transition.

HeLa cells were transfected with 20 nM control or DUE-B siRNA using Lipofectamine 2000 transfection reagent, and 24 hours later they were replated into new wells of 6-well plates to maintain a logarithmic growth state. Six hours after re-plate mimosine (500 μM) or aphidicolin (1 μg/ml) was added to the culture medium and the cells were allowed to arrest for 18 hours. Cells were then released from the cell cycle block for the indicated length of time and harvest for analysis by flow cytometry. The top panel shows an overlay of aphidicolin-arrested versus mimosine-arrested cells to demonstrate the subtle difference in the cell cycle profiles. Note that the scale on the y-axis is different in some of the panels in order highlight the differences in DNA content between samples.

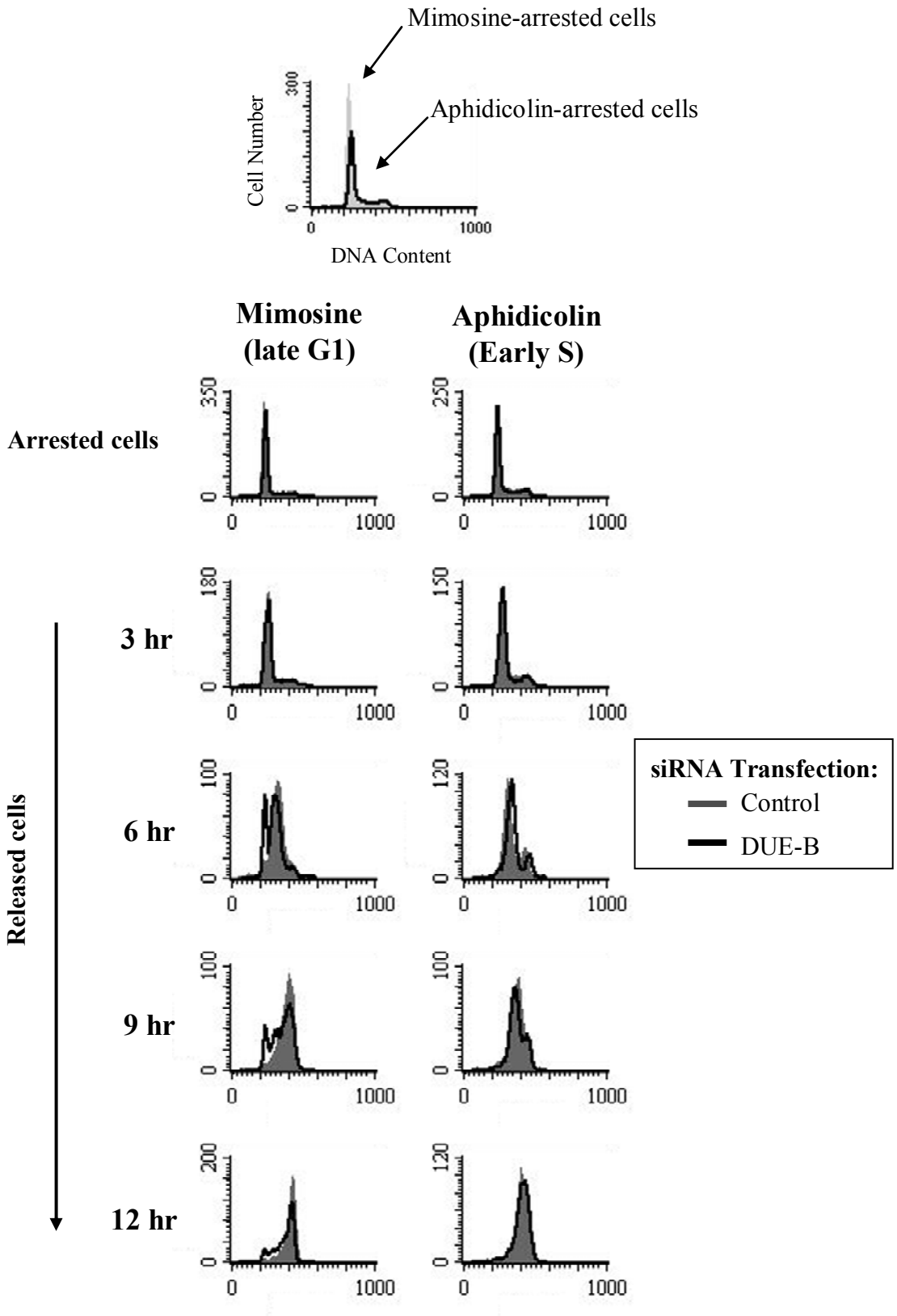

Mimosine-arrested cells
Aphidicolin-arrested cells
Cell Number
300
0
0
1000
DNA Content
Mimosine
(late G1)
Aphidicolin
(Early S)
Arrested cells
Released cells
3 hr
6 hr
9 hr
12 hr
350
250
180
150
100
120
100
100
200
120
siRNA Transfection:
Control
DUE-B

Figure 35. DUE-B RNAi has no effect on cells released from an early S phase arrest with hydroxyurea.

HeLa cells were transfected two times with 40 nM control or DUE-B siRNA over a period of 36 hours. Cells were then arrested at G1/S using a 20 hour treatment with 2 mM hydroxyurea (HU). Cells were then released from the cell cycle arrest for 5 hours and harvested for DNA content analysis by flow cytometry.

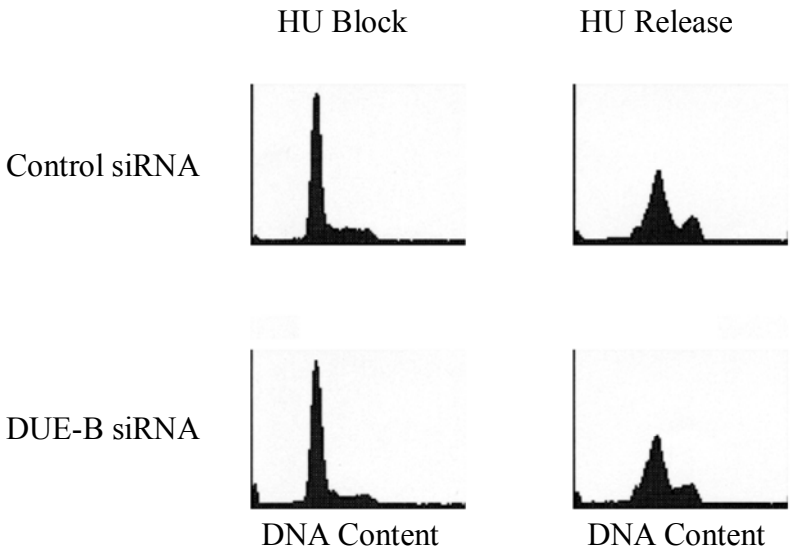
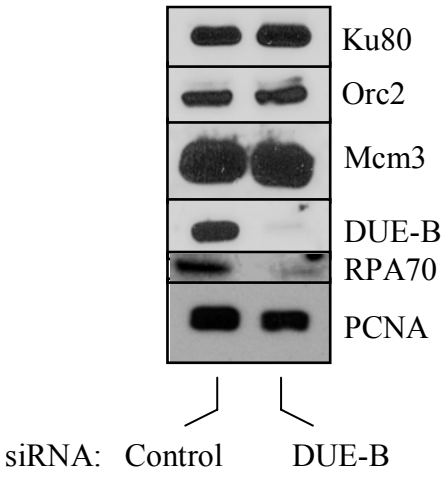

HU Block
HU Release
Control siRNA
DUE-B siRNA
DNA Content
DNA Content

Figure 36. DUE-B RNAi affects pre-RC activation but not pre-RC formation.

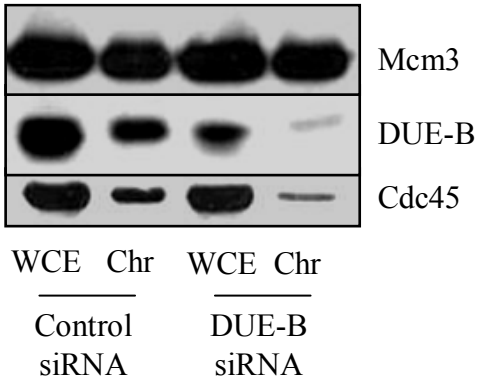
(A) HeLa cells at ~40% confluence in 15 cm tissue culture plates were transfected with 40 nM control or DUE-B siRNA using Lipofectamine 2000 reagent. After 24 hours, each 15 cm plate of cells was re-plated into three new 15 cm plates and allowed to grow an additional 24 hours. Cells were subsequently fractionated to yield a chromatin-enriched fraction for analysis by western blotting. (B) HeLa cells (~50% confluent 15 cm plate) were transfected with either 30 nM control or DUE-B siRNA using Lipofectamine 2000 reagent. Seven hours later each 15 cm plate was re-plated into four new 15 cm plates and allowed to grow overnight. The following day the cells were treated with 500 μM mimosine for 18 hours to arrest at late G1 phase of the cell cycle. Half the plates were subsequently used directly for preparation of whole cell extracts or for fractionation to enrich for chromatin-bound proteins in mimosine-arrested cells. The other plates of cells were then released from the mimosine block into fresh medium containing 1 μg/ml aphidicolin to allow RNA priming but block DNA synthesis and replication fork progression. After 7 hours the cells were harvested as for the mimosine-arrested cells. Whole cell extracts (WCE) or chromatin fractions (Chr) from an equivalent number of cells were then separated by SDS-PAGE and analyzed by western blotting.

A **Chromatin – asynchronous cells**



B

Release from mimosine into aphidicolin



effect on either Orc2 or Mcm3 levels on chromatin (Figure 36A), suggesting that DUE-B is not required for pre-RC formation. Interestingly the levels of both RPA70 and PCNA were reduced on chromatin depleted of DUE-B, arguing that DUE-B may be required for these proteins to bind to chromatin at replication forks. Both RPA70, a component of the heterotrimeric ssDNA binding protein complex, and PCNA, a clamp that increases the processivity of the replicative polymerases on DNA, bind replication origins after pre-RC activation.

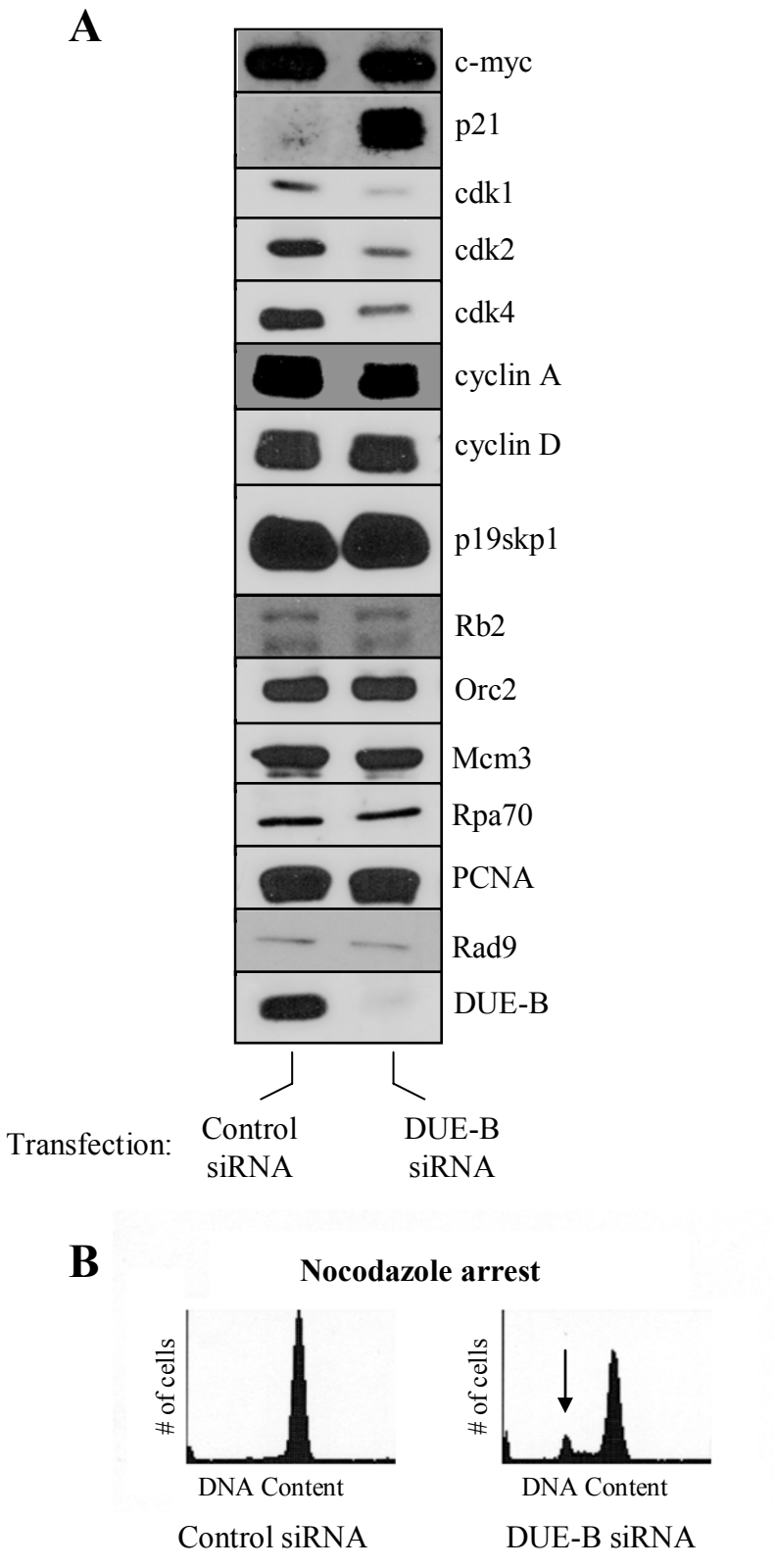
In order to delineate more clearly the step during initiation that DUE-B may act, HeLa cells transfected with either control or DUE-B siRNA were released from a block in late G1 with mimosine into medium containing aphidicolin, then fractionated to enrich for chromatin-bound proteins. These synchronizations should aid in the enrichment on chromatin of proteins that bind replication origins because of the differences in arrest points between mimosine and aphidicolin. Mimosine arrests cells in late G1 before the establishment of active replication forks, whereas aphidicolin arrests cells ~2 hours later (Krude, 1999; Lalande, 1990) by stalling active replication forks. Examination of chromatin-bound proteins from siRNA-transfected cells treated under these conditions showed a reduction of Cdc45 on chromatin in cells depleted of DUE-B (Figure 36B). Again, pre-RC formation was unaffected, as Mcm3 levels remained unchanged after depletion of DUE-B.

Thus both flow cytometric analyses and chromatin binding experiments support a model that the function of DUE-B at replication origins is to allow efficient activation of the origin by specifically promoting the recruitment of the MCM helicase co-factor Cdc45 or the ssDNA binding protein RPA onto chromatin.

To support the model that cell cycle progression is altered in cells depleted of DUE-B, whole cell extracts of HeLa cells transfected with either control or DUE-B siRNA were examined by western blot analysis. The total cellular expression of several replication origin binding proteins was unaffected by DUE-B depletion, including Orc2, Mcm3, RPA70, PCNA, and Rad9 (Figure 37A). Cyclins serve to regulate cyclin-dependent kinase (CDK) activity through the cell cycle, and we observed a small reduction in cyclin A, the S-phase cyclin, consistent with observations that DUE-B knock-down inhibits entry of cells into S phase. Interestingly, we observed a large increase in the CDK inhibitor protein p21. A primary transcriptional activator of p21 is the tumor suppresor p53, and previous data demonstrating that p53 levels

Figure 37. Effect of DUE-B RNAi on the expression of cell cycle-related proteins.

(A) HeLa cells were transfected at twice with 40 nM control or DUE-B siRNA (at time 0 and 24 hours) using Lipofectamine 2000, then harvested for analysis at 48 hours by western blot using antibodies against a variety proteins involved in DNA replication and/or cell cycle progression. (B) HeLa cells transfected as above were treated for 14 hours with nocodazole (100 ng/ml) 48 hours after the initial transfection. Mitotic cells that were floating in the medium were discarded, and only the attached cells were taken for analysis by flow cytometry to determine DNA content. The arrow indicates a population of cells transfected with DUE-B siRNA that are irreversibly arrested with a G1 phase DNA content.


A
c-myc
p21
cdk1
cdk2
cdk4
cyclin A
cyclin D
p19skp1
Rb2
Orc2
Mcm3
Rpa70
PCNA
Rad9
DUE-B
Transfection:
Control siRNA
DUE-B siRNA
B
Nocodazole arrest
# of cells
DNA Content
Control siRNA
# of cells
DNA Content
DUE-B siRNA

are unaffected in HeLa cells argues that this induction is p53-independent. We also observed decreases in cdk1, cdk2, and cdk4 protein levels, an unusual observation in that the primary mechanism of CDK regulation is through alteration of activity, by interaction with cyclins and proteins such as p21. Together these results are consistent with the observed alterations in cell cycle progression in cells depleted of DUE-B, though further work will be required to understand how and why these changes in protein levels are occurring.

The induction of p21 may be expected to result in an irreversible G1 arrest if the protein levels are high enough. Interestingly in some experiments we observed such a phenotype, typically in experiments in which DUE-B siRNA-transfected cells were not examined until at least three days after efficient DUE-B knockdown. Such an example is displayed in Figure 37B, where cells arrested with nocodazole showed a significant population of cells that remained in G1 phase, unable to progress through S phase and be arrested in mitosis. This was most apparent when harvesting cells that remain attached to the plate after the nocodazole arrest, since mitotic cells typically round up and come off the plate or are only loosely attached to the plate. These results suggest that in some cells, possibly with significantly reduced DUE-B protein levels, the G1 arrest that is observed is irreversible. Whether this is due only to the lack of DUE-B, or the alteration of other proteins involved in cell cycle regulation, is currently unknown.

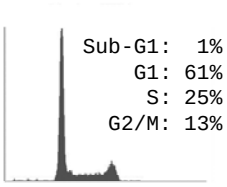
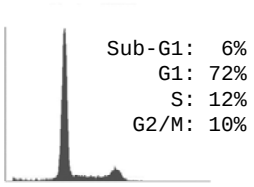
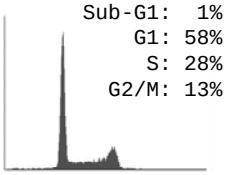
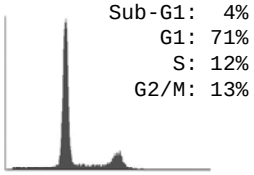
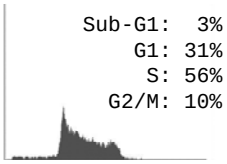
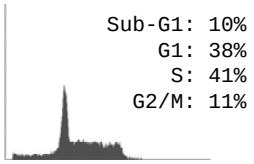
To test whether DUE-B was required for recovery from cell cycle arrest mediated by other means, HeLa cells were UV irradiated after transfection with a control or DUE-B siRNA. Cells depleted of DUE-B shows a significant enrichment in G1 cells 18 hours after recovery from UV treatment (Figure 38), suggesting DUE-B is required for restoration of DNA synthesis and entry into S phase after DNA damage.

In summary, the functional studies of DUE-B in human cells using RNAi implicate a role for DUE-B in the activation or formation of components of active replication forks, consistent with its isolation as a replication origin binding protein.

Figure 38. Effect of DUE-B RNAi on recovery from UV damage.

HeLa cells were transfected twice with 40 nM control or DUE-B siRNA using Oligofectamine transfection reagent (at time 0 and 43 hours), then irradiated with the indicated dose of UV seven hours after the second siRNA transfection. Cells were allowed to recover for 18 hours, then the remaining attached cells were fixed and processed for DNA content analysis by flow cytometry.

Transfection:

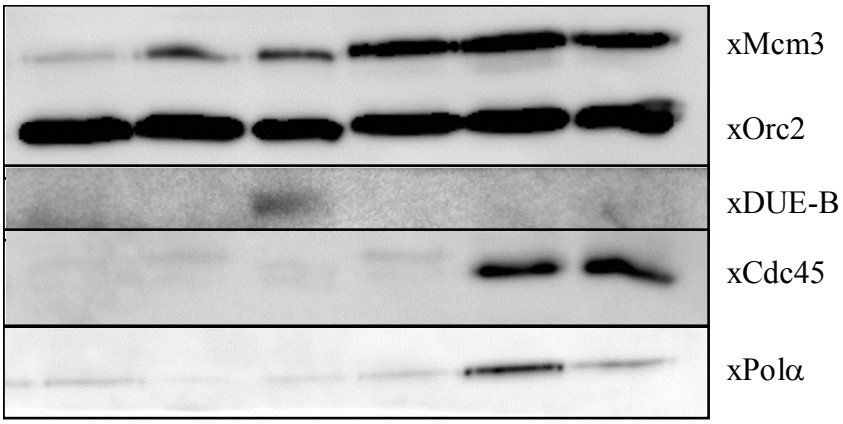
Treatment:	Control siRNA	DUE-B siRNA
No UV (Asynchronous)		
20 J/m^2 (18 hr recovery)		
100 J/m^2 (18 hr recovery)		

IV. Functional characterization of DUE-B in *Xenopus* egg extracts

Although the human DUE-B protein is homologous to proteins in organisms from bacteria to mammals, unique to vertebrate forms is a C-terminal extension of ~60 amino acids. Thus were we interested in using an additional vertebrate system besides cultured human cells to study the function of the DUE-B protein. Extracts prepared from the African clawed frog *Xenopus laevis* have served as a powerful system in which to investigate the regulation of the cell cycle, DNA replication, and other nuclear processes. As shown previously, antibodies raised against recombinant human DUE-B recognized a protein of the predicted molecular weight of xDUE-B in *Xenopus* egg extracts (Figure 16), and DNA binding assays have suggested the ability of xDUE-B to bind sperm chromatin (Figure 17). Since chromatin binding experiments in human cells had suggested hDUE-B binds to chromatin throughout the cell cycle, we were interested in examining the binding of xDUE-B to sperm chromatin in the *Xenopus* egg extract system. Because sperm chromatin does not contain any bound replication proteins prior to its incubation in an egg extract, it is a powerful tool for examining the regulated binding and assembly of replication complexes on DNA. Moreover, because the extracts are poised to undergo rapid cycles of DNA synthesis, replicon density is high and origin firing synchronous, unlike in somatic cells where origins initiate replication throughout an ~8 hour S phase. Sperm chromatin was added to a low-speed egg extract and at specific time intervals the reactions stopped and layered over a sucrose cushion for the enrichment of chromatin-bound proteins. As previously described, the ORC is the first replication initiation protein complex to assembly on sperm chromatin, and can be visualized on sperm chromatin within 5 minutes of placing S sperm DNA in the extract (Figure 39). Since ORC is required for loading of the MCM helicase on chromatin, its binding to DNA is slightly delayed relative to ORC, as demonstrated by xMcm3 beginning to bind by 10 minutes after addition of sperm chromatin to the extract. Mcm3 continued to load onto the DNA until approximately 30 minutes. At about this time point nuclei begin to form in the reaction, concentrating the kinases (CDK and DDK) required for pre-RC activation, thus promoting the initiation of DNA synthesis. The activation of pre-RCs can be defined as having taken place when Cdc45 and DNA polymerase alpha have been recruited to sperm chromatin, which occurred within the next 30 minutes in the reaction. Interestingly, very little DUE-B could be visualized on the chromatin, with the only apparent, stable binding occurring after 15 minutes of incubation of DNA in the extract. Although very little of the

Figure 39. xDUE-B binds sperm chromatin transiently during replication protein complex assembly in Xenopus egg extracts.

Sperm chromatin was added to a low-speed Xenopus egg extract (1800 sperm/μl) and after the indicated number of minutes the chromatin was purified, washed, and analyzed by SDS-PAGE and western blotting against the indicated proteins.


xMcm3
xOrc2
xDUE-B
xCdc45
xPolα
5
10
15
30
60
APH
Time (min) after addition of sperm chromatin to extract

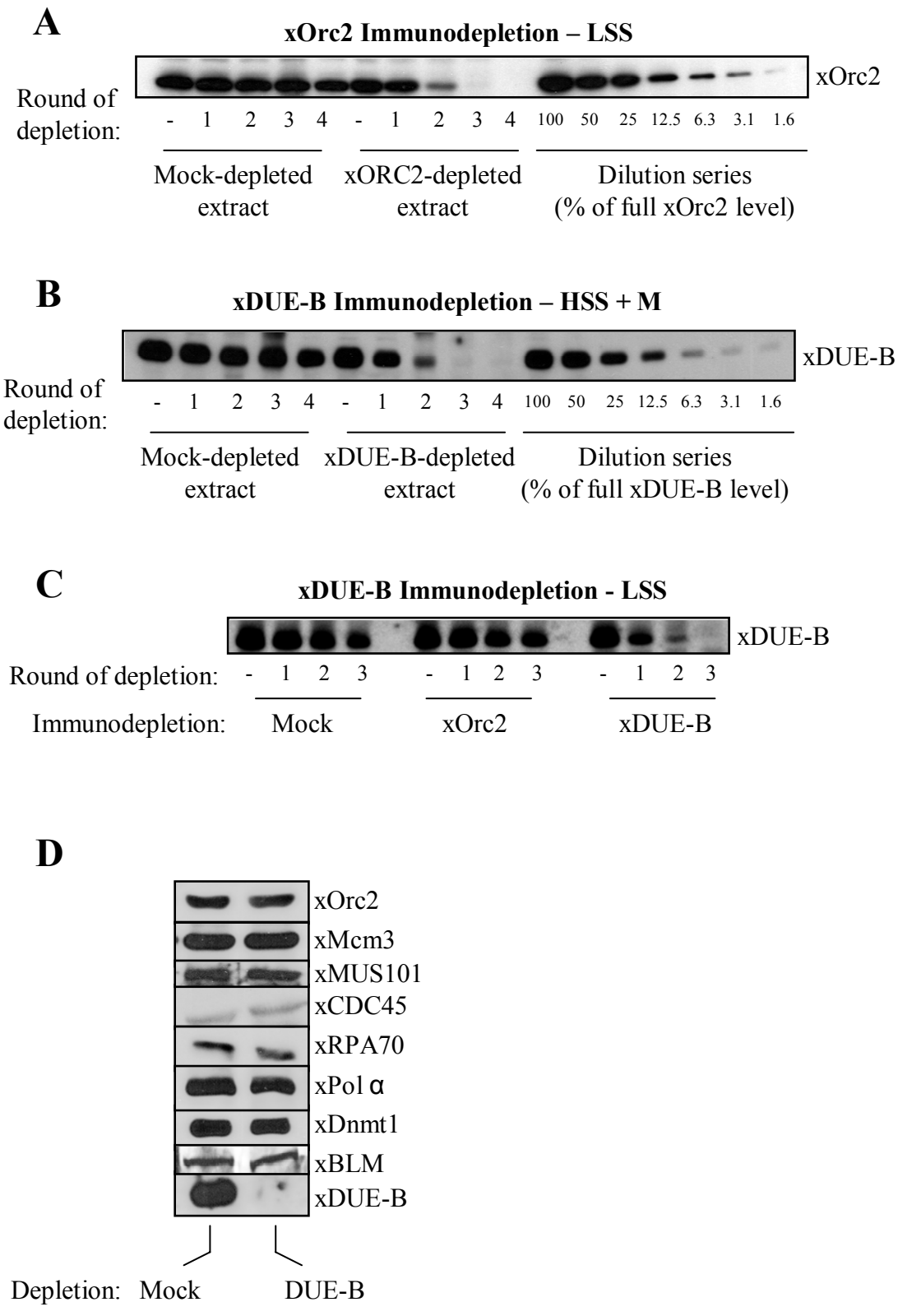
total amount of xDUE-B in the extract appears to bind to the DNA, it was repeatedly observed after roughly 15 minutes of incubation time. It may be that DUE-B binding to sperm chromatin is not a stable interaction and/or the methods used to purify the chromatin result in a loss of DUE-B signal on the chromatin. Consistent with the notion that DUE-B binding to sperm chromatin is unstable, preliminary experiments have indicated that formaldehyde crosslinking of proteins to sperm chromatin increases the detectable amount of xDUE-B on the DNA (data not shown). It is also possible that xDUE-B performs an enzymatic function at replication origins and only transiently interacts with each replication origin to perform its activity. The time point at which xDUE-B binds to sperm chromatin is consistent with RNAi data from human cells showing that DUE-B's role at replication origins may occur after the MCMs have begun to load at the origin. Further work will be required to determine precisely when xDUE-B functions in the assembly of replication complexes on sperm chromatin in *Xenopus* egg extracts. Interestingly, although the binding of rDUE-B^{Sf9} to sperm chromatin could be detected in the absence of *Xenopus* egg extract, an initial ELISA-based assay suggested that egg extract inhibited rDUE-B^{Sf9} binding to DNA, and similarly we have been unable to detect its binding of rDUE-B^{Sf9} to sperm chromatin in the presence of a *Xenopus* egg extract (data not shown). These results suggest that the binding of xDUE-B and rDUE-B^{Sf9} to DNA may be differentially regulated in egg extracts.

DUE-B can be specifically immunodepleted from egg extracts

To study the role of xDUE-B in the *Xenopus* egg extract replication system it was necessary to be able to deplete xDUE-B effectively from egg extracts. To do this, antibody-conjugated agarose beads were incubated several times with egg extract to remove the target protein, and the depleted supernatant used in replication assays. An example of this technique is displayed in Figure 40, showing that three rounds of incubation of low-speed egg extract with anti-xOrc2 antibody-bound agarose beads was sufficient to remove nearly all of xOrc2 from the extract (Figure 40A). Using a similar approach xDUE-B could be efficiently removed from either a high-speed egg extract (Figure 40B) or from a low-speed egg extract (Figure 40C). Three to four rounds of immunodepletion were typically required to deplete >99% of the xDUE-B from the extract. For depletion of the high-speed extract (HSS) it was necessary to first add the membrane fraction (M) to the extract, since some preparations of membranes contain significant amounts of xDUE-B (Casper, 2004). The immunodepletions were specific, as demonstrated by the inability of

Figure 40. xDUE-B and xORC2 can be immunodepleted from Xenopus egg extracts.

(A) Xenopus low-speed interphase egg extract (100 µl) was immunodepleted of xOrc2 using xOrc2 antibody-bound rProtein G-agarose beads. xOrc2 could be routinely be depleted by >99% by 3-4 rounds of immunodepletion (45 minutes each, 4°C). (B) Xenopus high-speed interphase egg cytosol (100 µl) and membrane fraction (10 µl) were combined and immunodepleted of xDUE-B using xDUE-B antibody-bound rProteinG-agarose beads. xDUE-B could be depleted by >99% after 3 rounds of immunodepletion (30 minutes each, 4°C). (C) Normal rabbit serum, anti-xOrc2 antiserum, and anti-DUE-B antiserum were coupled to rProtein G-agarose beads and then exposed to 100 µl of a low-speed, Xenopus interphase egg extract. Three rounds of depletion were performed, with each depletion taking place at 4°C for 30 minutes. A 0.5 µl aliquot of extract was taken after each round of depletion for western blot analysis to check the level of remaining xDUE-B in the extract. Three rounds of depletion are required to deplete xDUE-B in 100 µl of low-speed extract by >99%. (D) Mock- or xDUE-B-depleted egg extracts were separated by SDS-PAGE and analyzed by western blot to detect whether xDUE-B depletion has any effect on the levels of other replication/repair proteins in the extract.


A
xOrc2 Immunodepletion – LSS
xOrc2
Round of depletion:
- 1 2 3 4 - 1 2 3 4 100 50 25 12.5 6.3 3.1 1.6
Mock-depleted extract
xORC2-depleted extract
Dilution series (% of full xOrc2 level)
B
xDUE-B Immunodepletion – HSS + M
xDUE-B
Round of depletion:
- 1 2 3 4 - 1 2 3 4 100 50 25 12.5 6.3 3.1 1.6
Mock-depleted extract
xDUE-B-depleted extract
Dilution series (% of full xDUE-B level)
C
xDUE-B Immunodepletion - LSS
xDUE-B
Round of depletion: - 1 2 3 - 1 2 3 - 1 2 3
Immunodepletion: Mock xOrc2 xDUE-B
D
xOrc2
xMcm3
xMUS101
xCDC45
xRPA70
xPol α
xDnmt1
xBLM
xDUE-B
Depletion: Mock DUE-B

depletion to affect the level of xDUE-B remaining in the extract (Figure 40C). Similarly, xDUE-B depletion had no effect on the level of a variety of replication and repair proteins in the extract (Figure 40D). These results demonstrate that xDUE-B can be effectively removed from the extract via immunodepletion.

xDUE-B immunodepletion inhibits sperm chromatin replication

To examine the effect of xDUE-B immunodepletion, xOrc2 or xDUE-B were immunodepleted from a low-speed *Xenopus* egg extract. Sperm chromatin and ^{32}P-dCTP were then added to the extract in the absence or presence of the DNA polymerase inhibitor aphidicolin, and the reactions incubated for 120 minutes at room temperature. Replication products were visualized by running the proteinase K digested reactions on an agarose gel, followed by exposure of the dried gel to X-ray film. As shown in Figure 41, both xOrc2 and xDUE-B immunodepletion significantly inhibit the aphidicolin-sensitive replication of sperm chromatin.

To examine whether a similar result could be observed in the high-speed egg extract system, xDUE-B was immunodepleted from HSS + M (membrane) and then used in a sperm chromatin replication assay. A representative time course is shown in Figure 42A with the quantitation provided in Figure 42B. To be sure that the lack of replication was due to the specific removal of xDUE-B and not some other protein in the extract, a purified form of recombinant DUE-B from a clonal HeLa cell line expressing a 6xHis-tagged DUE-B cDNA (rDUE-B^{HeLa}) was added to replication assays. Addition of rDUE-B^{HeLa} to the xDUE-B-depleted egg extract was able to restore the replication of sperm chromatin to that found in undepleted extracts, suggesting rDUE-B^{HeLa} can complement xDUE-B. An excess amount of rDUE-B^{HeLa} relative to the normal endogenous amount of xDUE-B was required to fully restore replication to xDUE-B-depleted extracts (Figure 43A), suggesting that only a portion of the rDUE-B^{HeLa} may be in a replication-competent form. A summary of several experiments using the rDUE-B^{HeLa} supplementation is displayed in Figure 43B, and western blot analysis indicated that ~4-fold excess rDUE-B^{HeLa} was typically required to restore replication fully. It should be noted that because the 6xHis-tagged rDUE-B heterodimerized with the endogenously expressed DUE-B in HeLa cells, two bands are visualized on the western blot (Figure 43B).

Figure 41. xDUE-B depletion inhibits sperm chromatin replication to a level similar to that of xORC2 depletion.

Xenopus low-speed egg extract was with mock-, xOrc2-, or xDUE-B-depleted and then used incubated with sperm chromatin at 10,000 sperm/µl extract for 2 hours in the presence of ^{32}P-dCTP. Reactions took place for 2 hours at room temperature in the presence or absence of 50 µg/ml aphidicolin.. Reactions were then digested with proteinase K and loaded on a 0.8% agarose gel. The gel was dried and exposed to film. The graph on top shows a quantification of the intensity of the replication products. The approximate positions of the wells (origin) and a 23 kb DNA marker are indicated.

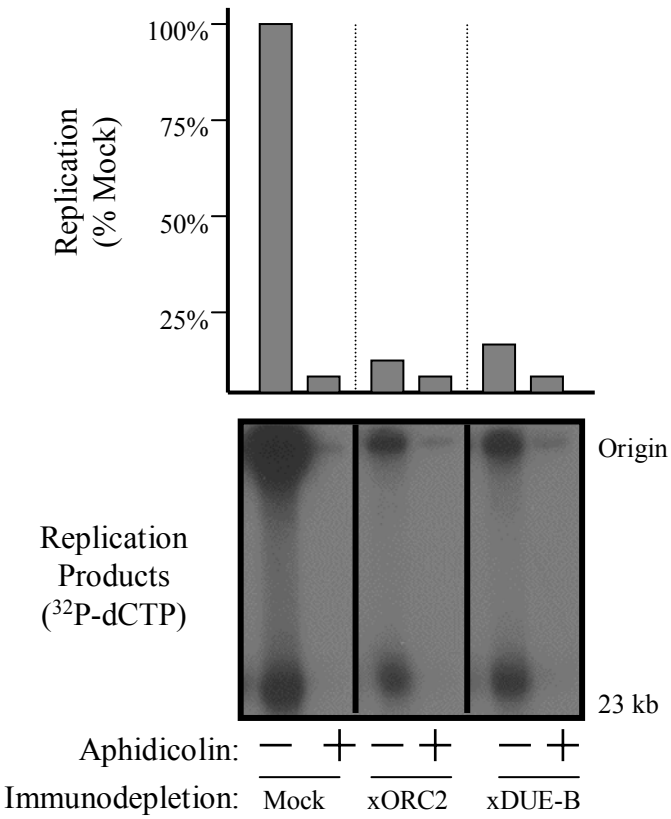
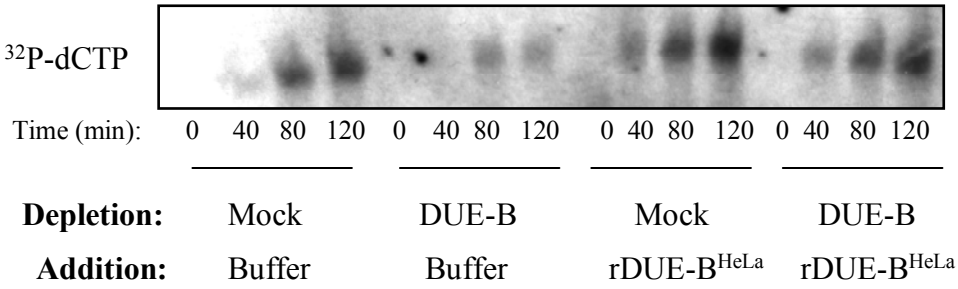

100%
75%
50%
25%
Replication
(% Mock)
Origin
Replication
Products
(32P-dCTP)
23 kb
Aphidicolin: − + − + − +
Immunodepletion: Mock xORC2 xDUE-B

Figure 42. xDUE-B function can be complemented by rDUE-B^{HeLa}.

Xenopus high-speed egg extracts were either mock- or xDUE-B-depleted and then used in a replication assay containing 1000 sperm/μl, ^{32}P-dCTP, and either rDUE-B^{HeLa} or buffer. The replication assays were digested with proteinase K, phenol-chloroform extracted, and electrophoresed on a 0.8% agarose gel in 1XTAE. The gel was dried and analyzed by autoradiography (A) and the replication band quantified (B). The data are normalized to that of the reaction with mock depleted egg extracts with addition of buffer.

A


32P-dCTP
Time (min): 0 40 80 120 0 40 80 120 0 40 80 120 0 40 80 120
Depletion: Mock DUE-B Mock DUE-B
Addition: Buffer Buffer rDUE-BHeLa rDUE-BHeLa

B

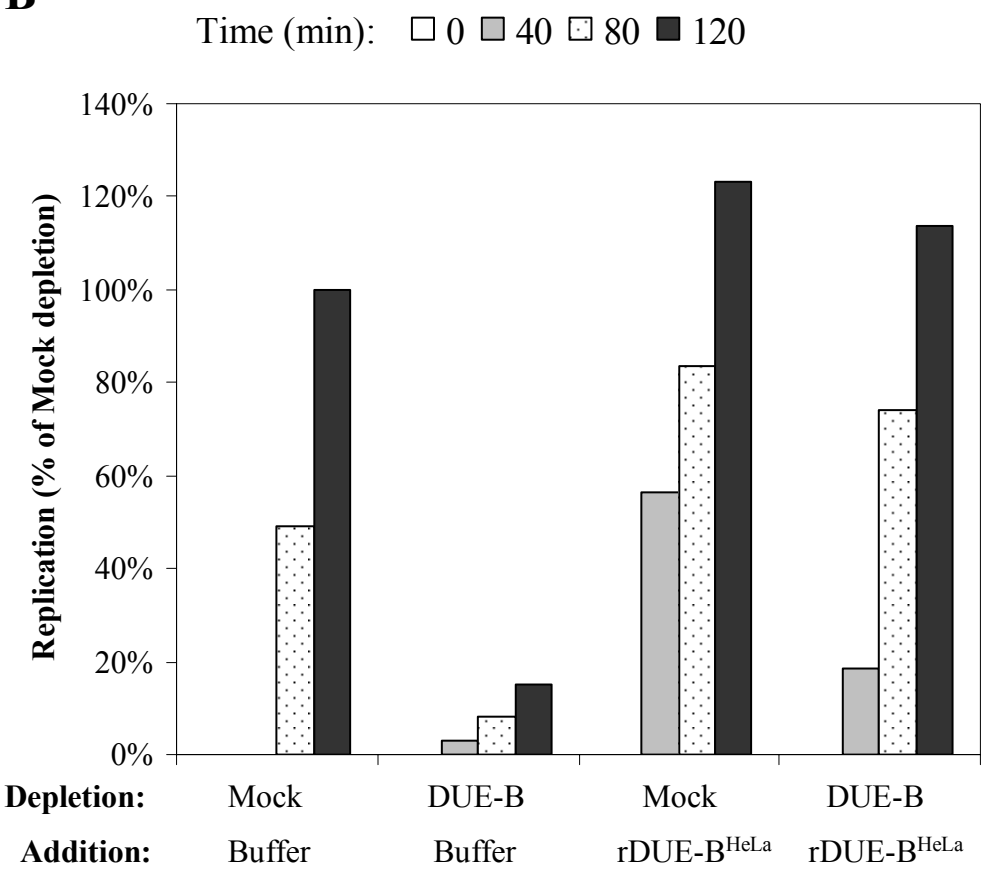
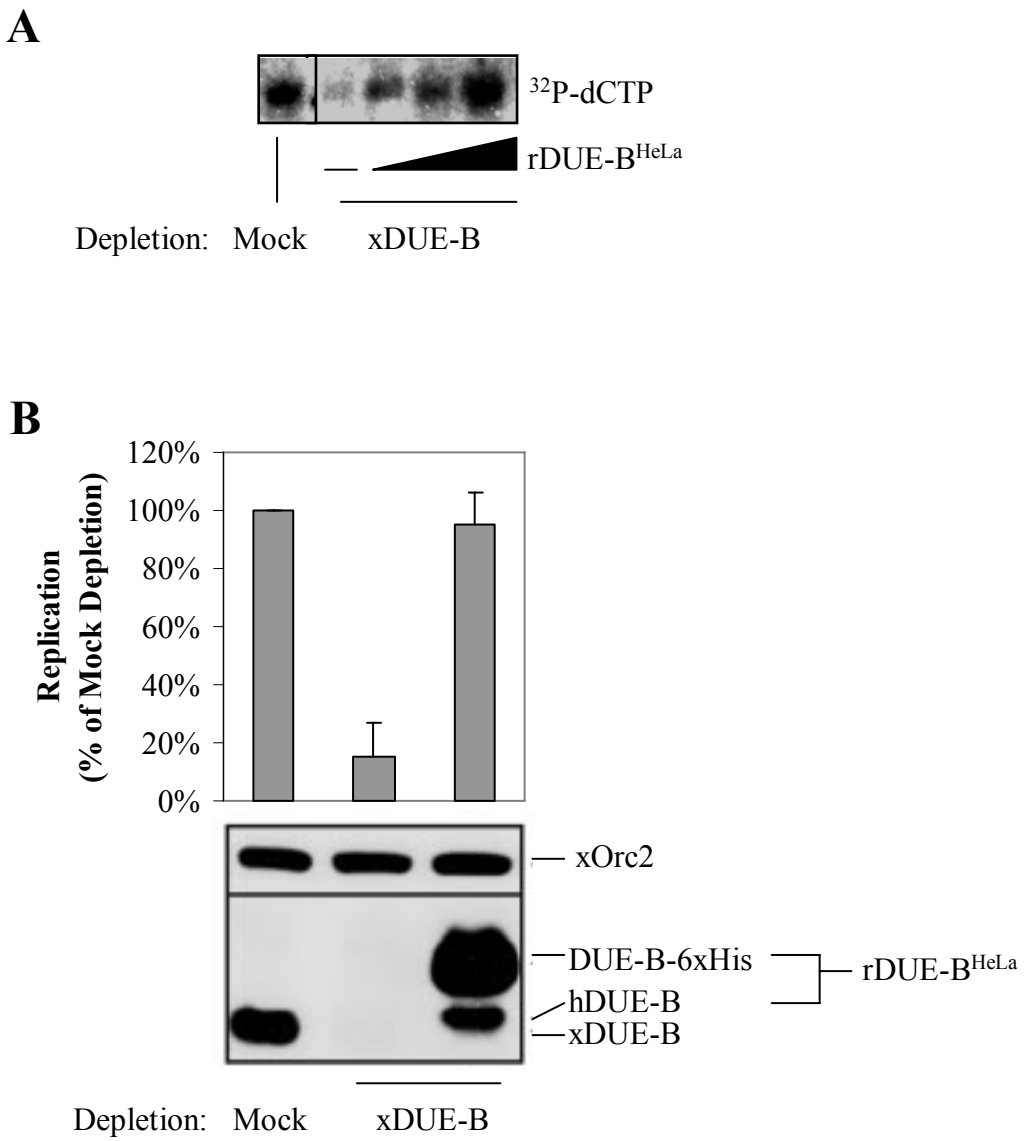

Time (min): 0 40 80 120
Replication (% of Mock depletion)
140%
120%
100%
80%
60%
40%
20%
0%
Depletion: Mock DUE-B Mock DUE-B
Addition: Buffer Buffer rDUE-BHeLa rDUE-BHeLa

Figure 43. The complementation of xDUE-B with rDUE-B^{HeLa} is dose dependent.

(A) Xenopus high-speed egg extract was either mock- or xDUE-B-depleted and then used in sperm chromatin replication assays containing 1000 sperm/µl. To the xDUE-B-depleted egg extract, either a 2.5-, 5-, or 10X-fold excess of rDUE-B^{HeLa} was added, relative to the level of xDUE-B normally found in the extract. The reactions were stopped at 120 minutes, digested with proteinase K, phenol-chloroform extracted, loaded on a 0.8% agarose gel, and analyzed by autoradiography. (B) Summary of experiments adding back rDUE-B^{HeLa} to xDUE-B-depleted egg extracts. Represented data are an average of 4-5 independent replication assays. Approximately a 4-fold excess of rDUE-B^{HeLa} was used to restore replication in xDUE-B-depleted egg extracts to the level found after 90 minutes in mock-depleted extracts.


A
32P-dCTP
rDUE-BHeLa
Depletion: Mock xDUE-B
B
Replication
(% of Mock Depletion)
120%
100%
80%
60%
40%
20%
0%
xOrc2
DUE-B-6xHis
hDUE-B
xDUE-B
rDUE-BHeLa
Depletion: Mock xDUE-B

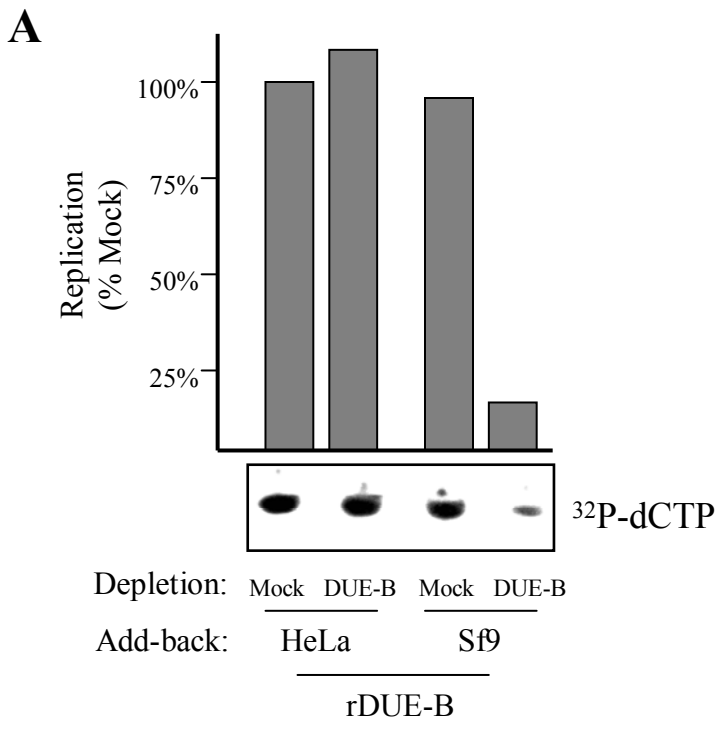
As previous data suggested that recombinant DUE-B from baculovirus-infected insect cells (rDUE-B^{Sf9}) inhibited sperm chromatin in the high-speed or nucleoplasmic extract replication systems, we wanted to test whether rDUE-B^{Sf9} could complement xDUE-B in egg extracts. High-speed extracts were immunodepleted of xDUE-B and then used in a sperm chromatin replication assay after addition of either rDUE-B^{HeLa} or rDUE-B^{Sf9}. We observed that the rDUE-B^{Sf9} was unable to complement xDUE-B and thus could not restore replication to the xDUE-B depleted egg extracts (Figure 44A). Interestingly, additional data further suggested a difference in activity between rDUE-B^{Sf9} and xDUE-B in egg extracts, with ^{32}P labeling showing robust, replication-stimulated phosphorylation of rDUE-B^{Sf9} but no phosphorylation of xDUE-B (Figure 44B). These results suggest that the two forms of DUE-B contain different patterns of post-translational modification. This difference in modification may therefore affect the ability of DUE-B to either stimulate or inhibit sperm chromatin replication, either directly in terms of DNA binding, or through an interaction with other accessory protein factors.

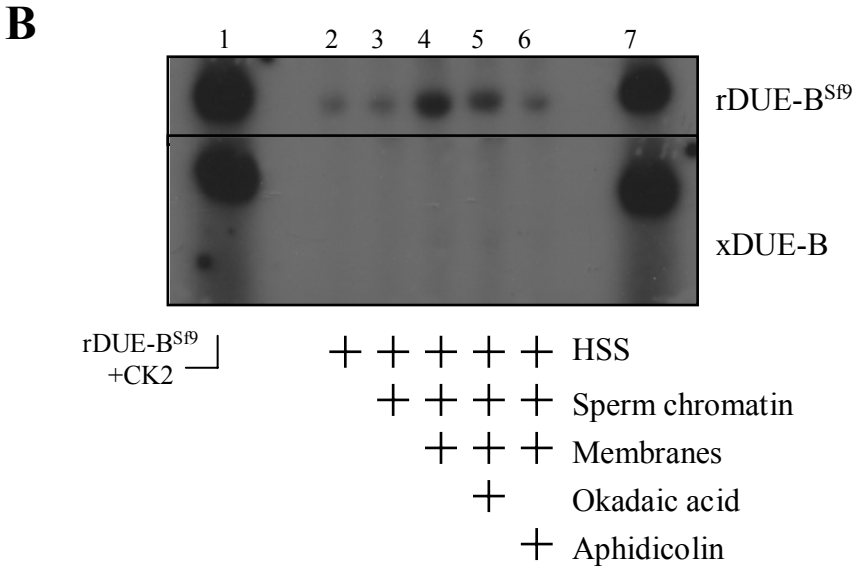
Summary

The utility of *Xenopus* egg extracts as a model system for studying the biochemistry of proteins involved in chromosomal DNA replication, coupled with data indicating an essential role for xDUE-B in duplication of the genome, warrant further use of egg extracts to investigate the precise stage of replication initiation at which DUE-B exerts its function. Data from human cells indicating a requirement for DUE-B in the binding of Cdc45 to chromatin and evidence showing rDUE-B^{Sf9} inhibits RPA loading on sperm chromatin in *Xenopus* egg extracts (Casper et al, 2005) together implicate a role for DUE-B in the establishment of active replication forks.

Figure 44. rDUE-B^{Sf9} cannot complement xDUE-B, and is differentially phosphorylated upon incubation in Xenopus egg extract.

(A) To mock (M)- or xDUE-B (D)-depleted high-speed Xenopus egg extracts (including membrane fraction), rDUE-B^{HeLa} or rDUE-B^{Sf9} was added and a sperm chromatin replication assay performed (120 minutes). The rDUE-B proteins are added at ~4-fold molar excess over that found in normal, undepleted egg extracts. (B) A Xenopus high-speed egg extract was incubated with or without rDUE-B^{Sf9} for 90 minutes at room temperatures in the presence of γ-^{32}P-ATP. Some of the reactions contained sperm chromatin (1500/µl) and membranes to allow formation of nuclei and DNA replication to occur. Some reactions contained either aphidicolin (50 µg/ml) or okadaic acid (1 µM) to inhibit DNA synthesis and pre-RC activation, respectively. xDUE-B and rDUE-B^{Sf9} (4-fold molar excess over xDUE-B levels) were then immunoprecipitated from the reactions and analyzed by SDS-PAGE and autoradiography. rDUE-B^{Sf9} phosphorylated with CK2 *in vitro* was used as a positive control for the identification of the rDUE-B^{Sf9} band. xDUE-B, which run slightly faster than rDUE-B^{Sf9} was unable to be detected on the autoradiogram, suggesting it does not become phosphorylated in egg extracts.


A
100%
75%
50%
25%
Replication
(% Mock)
32P-dCTP
Depletion:
Mock
DUE-B
Mock
DUE-B
Add-back:
HeLa
Sf9
rDUE-B


B
1
2
3
4
5
6
7
rDUE-BSf9
xDUE-B
rDUE-BSf9
+CK2
HSS
Sperm chromatin
Membranes
Okadaic acid
Aphidicolin

V. DUE-B is phosphorylated by CK2

rDUE-B^{HeLa} and rDUE-B^{Sf9} are differentially phosphorylated

The ability of rDUE-B^{HeLa} but not rDUE-B^{Sf9} to complement xDUE-B in *Xenopus* egg extracts (Figure 44A) and evidence showing that only rDUE-B^{Sf9} became phosphorylated in egg extracts (Figure 44B) together suggested a difference in activities between the different forms of DUE-B that may be attributed to a difference in phosphorylation. Since rDUE-B^{HeLa} can functionally replace xDUE-B we hypothesized that post-translational modifications on rDUE-B^{HeLa} recapitulate those found on endogenous xDUE-B present in interphase egg extracts. In an attempt to identify post-translational modifications on the two forms of recombinant DUE-B, purified rDUE-B^{HeLa} and rDUE-B^{Sf9} (Figure 45A) were analyzed by western blotting with an anti-phosphoserine antibody. Interestingly, rDUE-B^{HeLa} showed greater reactivity to the antibody than did rDUE-B^{Sf9}, suggesting an increase in the number of phosphoserine residues (Figure 45B).

To confirm an apparent increase in the number of phosphorylated serines and to try to identify specific sites of phosphorylation, rDUE-B^{HeLa} and rDUE-B^{Sf9} were digested in-gel with trypsin and analyzed by MALDI-TOF mass spectrometry. Although only a few tryptic peptide masses could be detected, one peptide mass (1578.593 Da) unique to the rDUE-B^{HeLa} sample was observed (Table 5). The other masses obtained were found in both samples. Interestingly this 1578.593 Da peptide is precisely 240 Da heavier than a mass of 1338.410 Da found in both samples. This smaller mass is predicted to correspond to a tryptic peptide between amino acids 194 and 207 in the C-terminal region of DUE-B. This peptide contains five serine residues, each of which has a high probability of being phosphorylated (Figure 45A) and is conserved in vertebrate homologs of DUE-B (Figure 46B). Because one phosphate group would account for an 80 Da increase in mass to the peptide, the 240 Da difference in mass between the two peptide masses is consistent with the presence of three phosphorylated serine residues on this peptide in the C-terminus of rDUE-B^{HeLa}. This difference in phosphorylation may account for the apparent difference in reactivity to a phosphoserine antibody by western blot analysis (Figure 45B).

Although we have been unable to define which three of the five serines are phosphorylated on rDUE-B^{HeLa}, we observed that this peptide contained three potential sites predicted to be targets of casein kinase II (CK2) (Figure 46B). Although the C-terminus of DUE-B is rich in basic amino acids (pI of the

Figure 45. Purified rDUE-B^{HeLa} reacts stronger than rDUE-B^{Sf9} to an anti-phosphoserine antibody. (A) Coomassie-stained gel of purified rDUE-B^{HeLa} and rDUE-B^{Sf9}. (B) 500 ng of each of the two forms of DUE-B were run on SDS-PAGE and analyzed by western blot analysis with an anti-phosphoserine antibody (Qiagen) or anti-DUE-B antisera. For the anti-DUE-B blot, only 10 ng of each protein was run on SDS-PAGE. (C) rDUE-BHeLa was treated with lambda phosphatase (λ-PPase) for 30 minutes and analyzed by SDS-PAGE and western blotting.

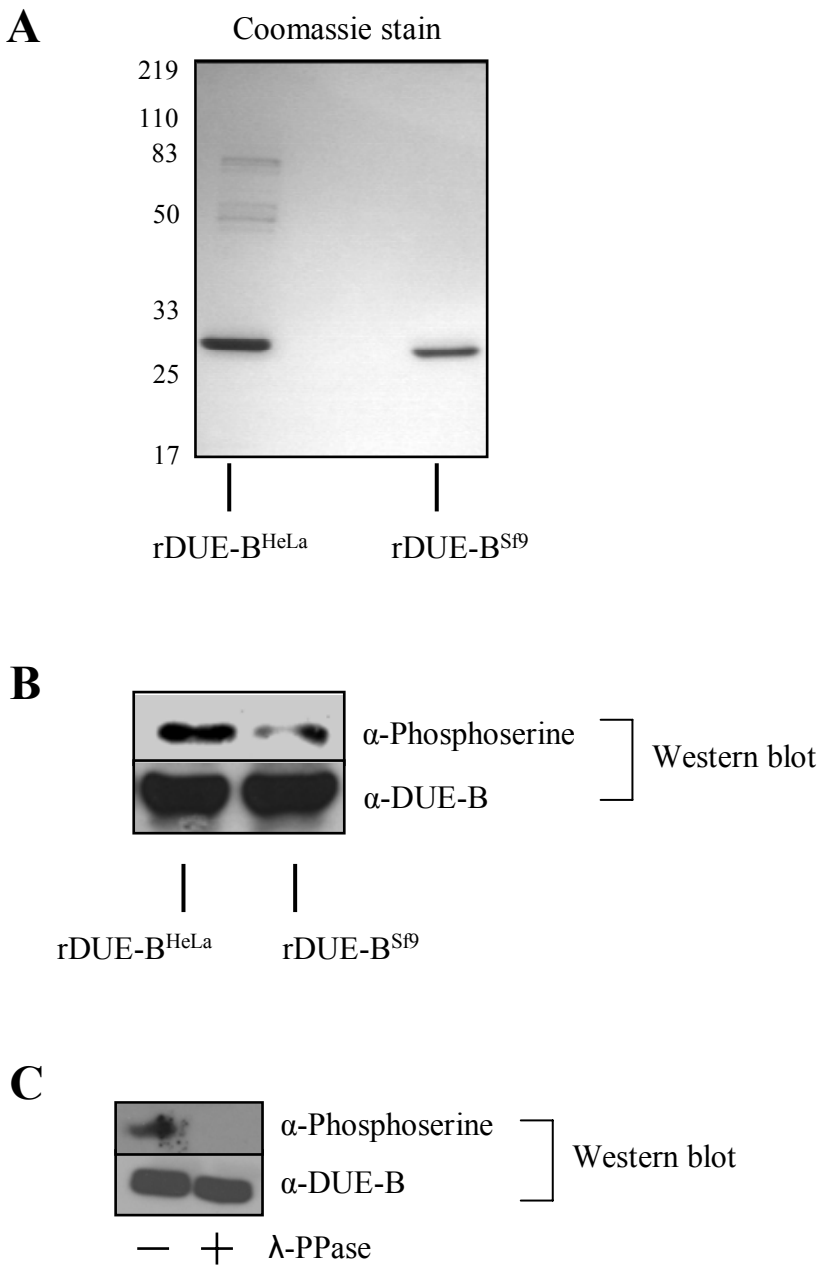

A
Coomassie stain
219
110
83
50
33
25
17
rDUE-B^HeLa
rDUE-B^Sf9
B
α-Phosphoserine
α-DUE-B
Western blot
rDUE-B^HeLa
rDUE-B^Sf9
C
α-Phosphoserine
α-DUE-B
Western blot
— + λ-PPase

Table 5. Summary of MALDI-TOF analysis of rDUE-B^{HeLa} and rDUE-B^{Sf9}.

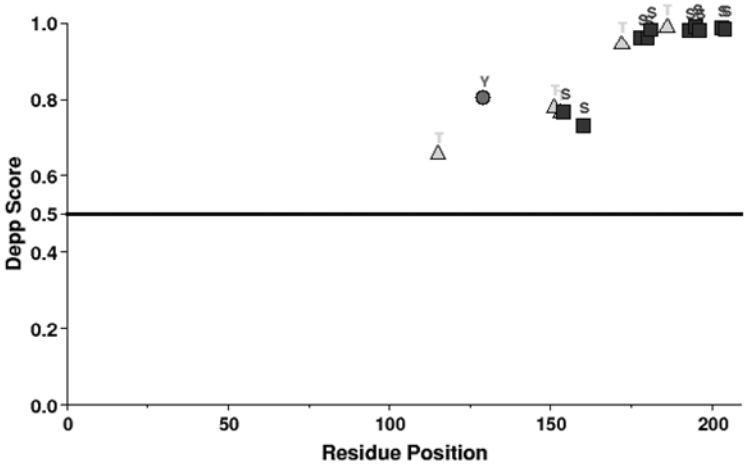
Coomassie-stained bands of rDUE-B^{HeLa} and rDUE-B^{Sf9} were digested with trypsin in-gel and analyzed by MALDI-TOF by Dr. Detlef Schumann (Genome Research Institute, Cincinnati, Ohio). The list of peptides corresponding to each form of rDUE-B is indicated. The predicted peptide modification(s) are listed.

rDUE-B^{HeLa}	rDUE-B^{Sf9}	Peptide Sequence	Mod
1267.628	1267.628	MKAVVQRVTR (1-10)	1 PO_4
1338.410	1338.410	SASSGAEGDVSSER (194-207)	-
1444.651	1444.651	ASVTVGGEQISAIGR (11-25) or GPSESSKERNTPR (177-189)	-
1578.593	-	SASSDAEGDVSSER (194-207)	3 PO_4

Figure 46. The C-terminus of DUE-B is predicted to be phosphorylated and contains several evolutionarily conserved CK2 phosphorylation sites.

(A) Using the algorithm DEPP (http://pondr.com) the DUE-B sequence was analyzed for the probability that any serine, threonine, or tyrosine is phosphorylated. Note that the serines and threonines in the C-terminus show the highest probability of being phosphorylated. (B) A C-terminal peptide of rDUE-B^{HeLa} identified as possibly containing three phosphates by MALDI-TOF analysis contains three potential target sites for caseine kinase 2 (CK2) that conserved in other vertebrates (in bold and underlined).

A



B

```
Human       --SASSGAEGDVSSEREP
Mouse       --SASSGAEGDVSSEREP
Chicken     --SASSGAEGDVSSEREP
Frog        --SASSGAEGDVSSEREP
Zebrafish   --SASSGAEGDVSSEREP
```

C-terminal 53 amino acids can be further segregated into a basic patch and an acidic patch. Amino acids 157-191 of DUE-B show a basic pI of 10.8, whereas amino acids 191-209 have a predicted pI of 4.1. The C-terminal tryptic peptide (amino acids 194-207) implicated as being phosphorylated lies in this latter acidic region, making it an ideal theoretical target for CK2 which preferentially phosphorylates proteins in acidic regions. Ser205 and Ser197 both show strong homology to the consensus site for CK2 phosphorylation, and CK2 would also likely target Ser194 in cases where Ser197 is phosphorylated, making the +3 amino acid acidic.

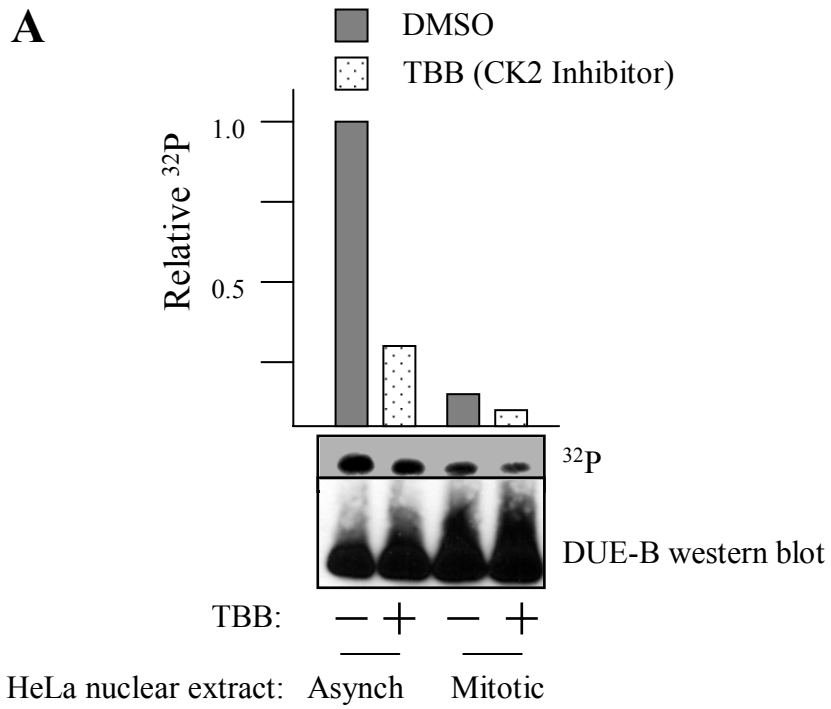
To determine whether DUE-B is a target of CK2, rDUE-B^{Sf9} was added to a HeLa nuclear extract prepared from either asynchronous or mitotic cells preincubated or not with the CK2 inhibitor 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB). Interestingly, even in the absence of TBB, rDUE-B^{Sf9} showed a striking difference in phosphorylation between the two extracts, with significantly less phosphorylation occurring in the mitotic extract (Figure 47A). These results confirm previous orthophosphate labeling studies in which endogenous DUE-B showed very low levels of phosphorylation in cells arrested in mitosis with nocodazole, compared to either asynchronous or S phase arrested cells. The significant reduction in phosphorylation that was observed in extracts containing the CK2 inhibitor TBB argued that the observed phosphorylation is CK2-dependent.

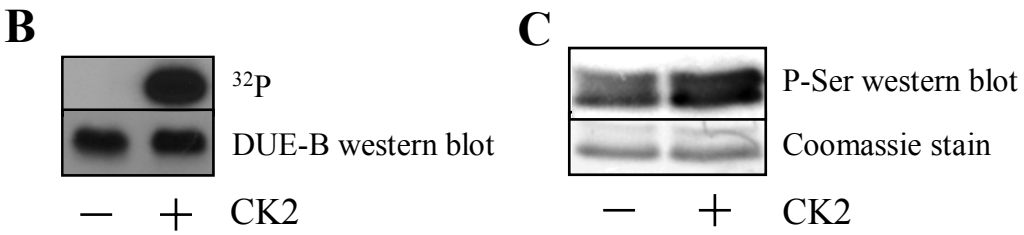
CK2 phosphorylates DUE-B *in vitro*

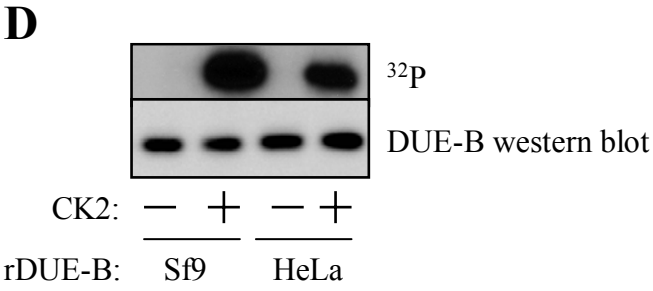
To determine whether CK2 can directly phosphorylate DUE-B, rDUE-B^{Sf9} was incubated in a reaction with [γ-^{32}P]-ATP and recombinant CK2 (rCK2). Robust phosphorylation was observed (Figure 47A) that increased the reactivity of rDUE-B^{Sf9} to a phosphoserine antibody (Figure 47C). Since both rDUE-B^{HeLa} and rDUE-B^{Sf9} contained the putative unphosphorylated C-terminal peptide mass (Table 5), we were interested in comparing the ability of recombinant CK2 (rCK2) to phosphorylate the two forms of rDUE-B *in vitro*. Interestingly rDUE-B^{Sf9} showed more labeling than rDUE-B^{HeLa} (Figure 47D), consistent with the mass spectrometry data showing rDUE-B^{HeLa} to contain a mixture of both phosphorylated and unphosphorylated peptides (Table 5). That rDUE-B^{HeLa} is only partially phosphorylated at the C-terminal peptide may account for the requirement of excess rDUE-B^{HeLa} to restore replication to xDUE-B-depleted egg extracts.

Figure 47. DUE-B is phosphorylated by CK2 *in vitro*.

(A) Phosphorylation of rDUE-B^{Sf9} in HeLa is CK2 dependent. HeLa nuclear extracts (150 μg) prepared from either asynchronous or mitotic cells were supplemented with 1 mM sodium orthovanadate, 20 mM sodium fluoride, 100 μM ATP, and 0.1 μCi/μl γ-^{32}P-ATP and then preincubated for 10 minutes with either DMSO or 5 μM TBB (Calbiochem). rDUE-B^{Sf9} (1.5 μg) was subsequently added to the extract for 1 hour at 30°C before incubation stopping the reaction with stop buffer (450 mM NaCl, 0.5% NP-40, 2 mM ATP) and incubating for 2 hours at 4°C with Ni-NTA agarose beads to affinity precipitate the rDUE-B from the extract. 90% of the precipitated material was run on SDS-PAGE for autoradiographic analysis, and the other 10% was analyzed by western blotting to determine the efficiency of DUE-B recovery. The ^{32}P signal was then normalized to the amount of DUE-B precipitated, and the ratios compared relative to the amount of rDUE-B precipitated in asynchronous extracts in the absence of TBB (top graph). (B) CK2 phosphorylates rDUE-B^{Sf9} directly *in vitro*. 750 ng rDUE-B^{Sf9} was incubated with 50 U CK2 for 30 minutes at 30°C in the presence of γ-^{32}P-ATP before stopping the reaction with SDS-PAGE sample buffer and analysis by autoradiography. 5% of the reaction was analyzed on a separate gel for western blotting with anti-DUE-B antisera. (C) rDUE-BSf9 (750 ng) was incubated with 50 U CK2 for 45 minutes at 30°C. The reaction was stopped by addition of SDS-PAGE sample buffer, and the reaction was electrophoresed on an SDS gel, transferred to a PVDF membrane, and analyzed by western blotting with an anti-phosphoserine antibody (Qiagen). The blot was subsequently stained with coomassie. (D) rDUE-B^{Sf9} is a better substrate than rDUE-B^{HeLa} for CK2. Equal amounts of rDUE-B^{Sf9} and rDUE-B^{HeLa} were phosphorylated with CK2 in reactions containing γ-^{32}P-ATP for 30 minutes at 30°C. Reactions were stopped by boiling in SDS-PAGE sample buffer and then 95% was loaded on a 12% SDS-polyacrylamide gel for autoradiography and 5% for western blot analysis.


A
DMSO
TBB (CK2 Inhibitor)
Relative 32P
1.0
0.5
32P
DUE-B western blot
TBB: — + — +
HeLa nuclear extract: Asynch Mitotic


B
32P
DUE-B western blot
— + CK2
C
P-Ser western blot
Coomassie stain
— + CK2


D
32P
DUE-B western blot
CK2: — + — +
rDUE-B: Sf9 HeLa

CK2 phosphorylates the C-terminal region of DUE-B

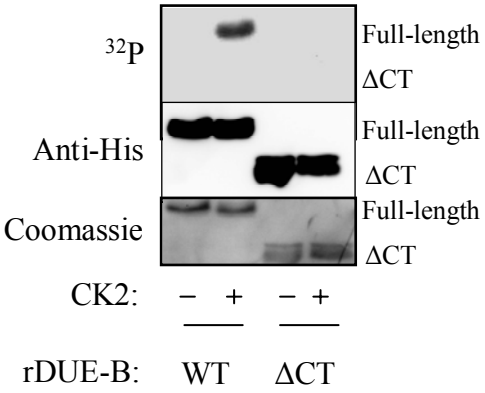
Since these experiments do not demonstrate the particular region of DUE-B targeted by CK2, a baculovirus construct was prepared to express a recombinant form of DUE-B lacking the C-terminal 62 amino acids. The two forms of rDUE-B^{Sf9}, termed either WT for the full-length construct and ΔCT for the C-terminal truncation mutant, were subsequently incubated with rCK2. Whereas rDUE-B-WT was efficiently phosphorylated, rDUE-B-ΔCT showed no labeling with ^{32}P (Figure 48A). Although these results strongly argue that the C-terminus of DUE-B is required for CK2 to phosphorylate DUE-B, it remained possible that rCK2 could still phosphorylate DUE-B within the N-terminal ~150 amino acids if the C-terminus was present. In an attempt to clarify this issue, rDUE-B-WT phosphorylated *in vitro* with rCK2 was exposed to 75% formic acid overnight. Formic acid cleaves proteins specifically between aspartate and proline (Piszkiewicz et al, 1970) and DUE-B contains one such predicted target site at the Asp156-Pro157 bond. The portion of the formic acid digest of rDUE-B that accounts for the N-terminal fragment is referred to as "core" in Figure 48B, and it retains reactivity to DUE-B antisera by western blot analysis but not reactivity to an anti-His antibody, since the C-terminus has been cleaved off. Because of the relatively small size of the C-terminal fragment, it may not be efficiently retained on the PVDF membrane used for western blotting. Very little incorporation of ^{32}P could be visualized as occurring in the N-terminal formic acid fragment, further arguing that CK2 phosphorylates DUE-B specifically in the C-terminus (Figure 48A).

Although mass spectrometry results did not pinpoint the specific sites of phosphorylation, the presence of three CK2 target sites within the C-terminal peptide and *in vitro* data demonstrating CK2 phosphorylates DUE-B within its C-terminus argue that CK2 may be a major kinase that regulates DUE-B function *in vivo*. Interestingly, the major kinase that phosphorylates the ORC in *Drosophila* extracts is also CK2, resulting in altered DNA binding characteristics (Remus et al, 2005). It is possible that CK2 plays a similar role in *Xenopus* egg extracts, though few studies are available that have dealt with CK2 in *Xenopus* egg extracts. fsFurther experimentation will be required to test the physiological relevance of CK2 phosphorylation of DUE-B.

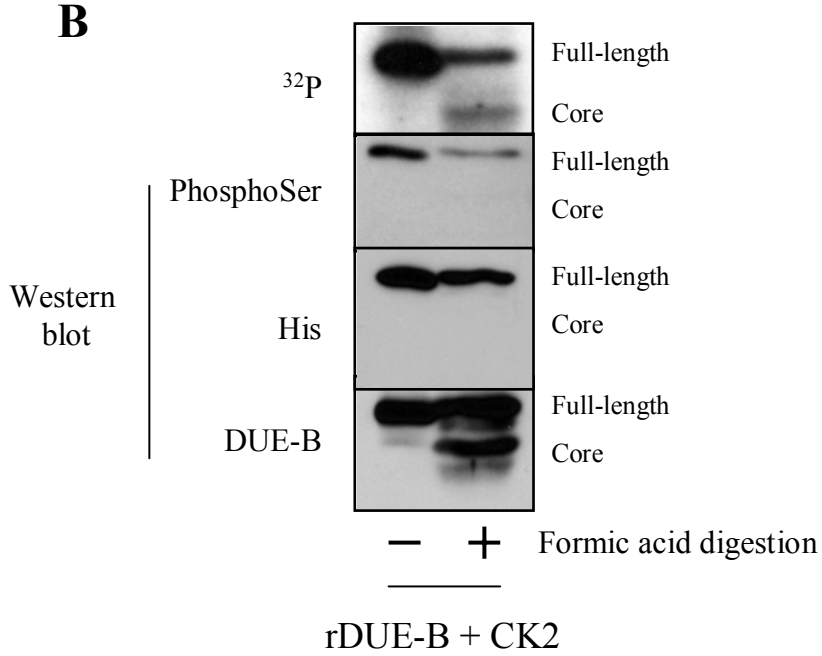
Figure 48. DUE-B is phosphorylated by CK2 primarily within the C-terminal domain.

(A) 1 μg of either rDUE-B-WT or rDUE-B-ΔCT were incubated in reactions containing CK2 (50 U) and γ-^{32}P-ATP (2 μCi) for 45 minutes at 30°C, then electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane analyzed by autoradiography and by western blot with an anti-6xHis antibody (Invitrogen). Finally the membrane was stained with coomassie as a second control for equal loading. (B) rDUE-B^{Sf9} (1.5 μg) was incubated in a 50 μl reaction with CK2 (50 U) and γ-^{32}P-ATP (5 μCi) for 30 minutes at 30°C. The reaction was then split into two aliquots (25 μl each), and either 75 μl water or 75 μl formic acid (75% final) was added. The reactions were then incubated overnight at 37°C. The sample treated with formic acid was then lyophilized and neutralized with NaOH. Both samples (with and without formic acid digestion) were split into aliquots for autoradiography or for western blot analysis against using the indicated antibodies.

A


32P
Full-length
ΔCT
Anti-His
Full-length
ΔCT
Coomassie
Full-length
ΔCT
CK2: − + − +
rDUE-B: WT ΔCT

B


Western blot
32P
Full-length
Core
PhosphoSer
Full-length
Core
His
Full-length
Core
DUE-B
Full-length
Core
− + Formic acid digestion
rDUE-B + CK2

VI. Structural characterization of DUE-B

In an effort to elucidate the role of DUE-B in DNA replication, a crystal structure of the human DUE-B protein was obtained in collaboration with the laboratory of Dr. Satish Nair (Figure 49). The 2.0 Å resolution structure corresponds to the amino terminal 151 amino acids of the full-length recombinant protein from baculovirus-infected insect cells. The structure also revealed the protein to be dimeric, consistent with solution studies (Casper et al, 2005) (Figure 49A and B). Residues Lys2 through Val15 form a long, extended β strand that is sharply bent at residue Thr9. Residues Glu18 through Ile23 and residues Gly26 through Gly32 form two additional β strands that comprise the core of the β-sheet structure. An α helix is formed by residues Gln39 through Asn51 and is followed by another strand of the β-sheet structure (Glu73 through Ser78). A second long helix encompases residues Thr99 to Thr116 and is is followed by a short β strand (Ile122 through Asp124). The final β strand is long and is formed by residues Met131 through Glu146, with a kink at residue Gly139. Each monomer of DUE-B forms a tight interacting dimer, with the interface mainly occurring through an antiparallel β-sheet structure created by interactions between residues Met131 to Gly139 of each monomer.

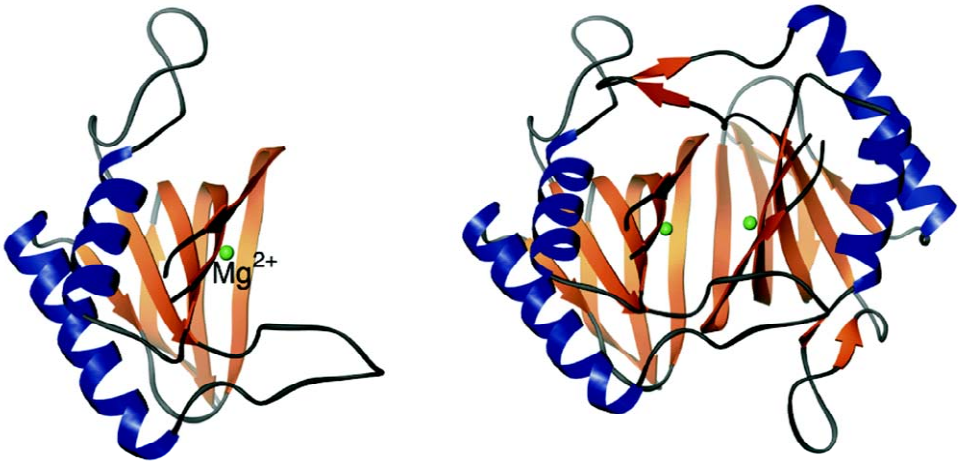
Interestingly the arrangement of amino acids in the DUE-B monomer resembled topologically many of the structural elements of polypeptides that contain nucleotide-binding motifs (Figure 50). Structure-based comparison of human DUE-B with that ATP binding protein MJ0577 (IMJH) yielded a root mean square deviation of 3.7 Å over 79 amino acids (Figure 50B). Superposition of 62 alpha carbon atoms common to both DUE-B and liver alcohol dehydrogenase yielded a root mean square of 3.7 Å, demonstrating that the DUE-B amino-terminal domain structure is similar to half of the canonical Rossman fold (Figure 50C). Since previous results had indicated that recombinant DUE-B possessed ATPase activity, it is likely that the structural similarity between the N-terminal domain of DUE-B and these proteins extends to function as a nucleotide binding motif.

During the initial phase of this work, structures of proteins with significant sequence similarity to DUE-B, including both the *E. coli* and *H. influenzae* D-aminoacyl-tRNA deacylases, were obtained and published by other groups (Ferri-Fioni et al, 2001; Lim et al, 2003). Interestingly, the structure we obtained of DUE-B showed significant structural similarity to these bacterial proteins (Figure 51A and B), as well as to a

Figure 49. Crystal structure of DUE-B.

(A) Ribbon diagrams of the DUE-B monomer (left) and dimer (right), with magnesium ion(s) shown in green. This image was generated and provided by Dr. Satish Nair. (B) Image of DUE-B dimer generated using the Swiss Protein Database Viewer (SPDBV), rotated so that the C-termini of the crystalizable regions are at the bottom of the structure.

A



B

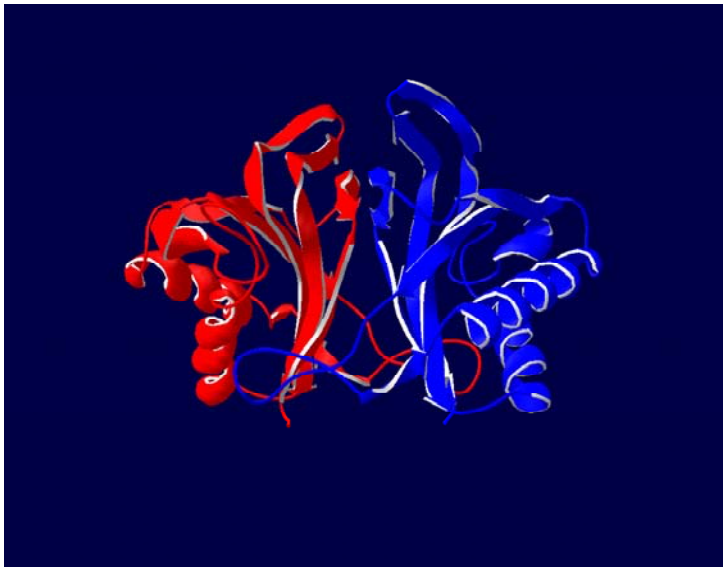


Figure 50. Structural similarities of DUE-B and ATP binding proteins.

The three-dimensional structure of DUE-B (A) shows similarity to proteins that contain nucleotide-binding motifs, such as the ATP binding protein MJ0577 (1MJH) (B) and liver alcohol dehydrogenase (C).

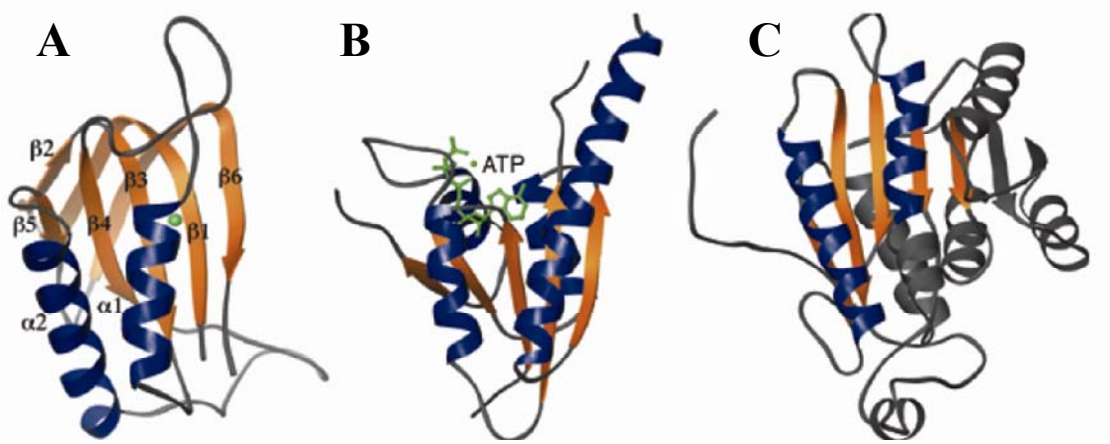
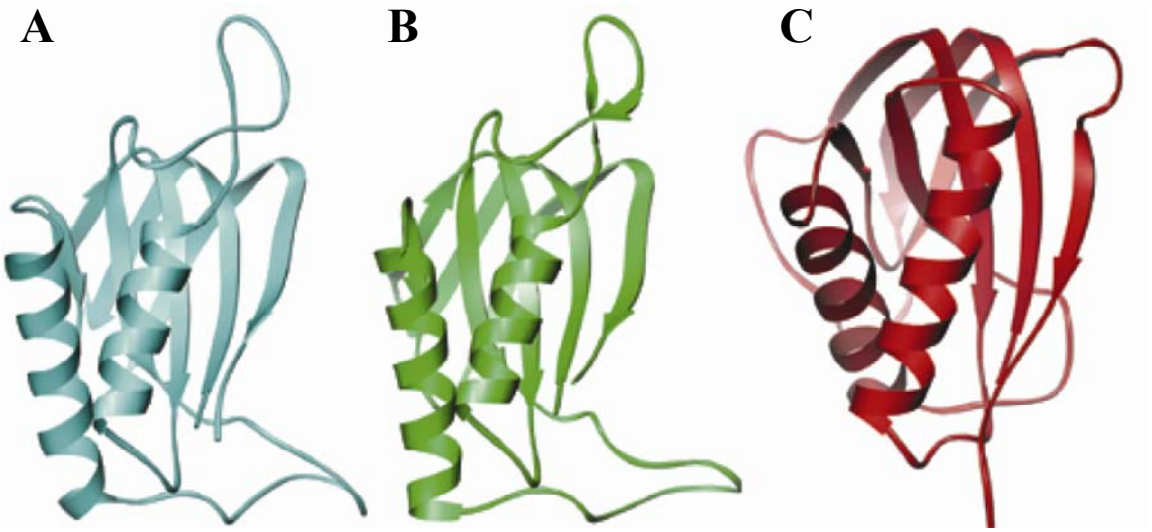

A
B
C
β2
β3
β6
β5
β4
β1
α2
α1
ATP

Figure 51. Conservation of DUE-B structure.

Ribbon diagrams of human DUE-B (A), the *E. coli* D-Tyr-tRNATyr deacylase (B), and the archaea-specific editing domain of threonyl-tRNA synthetase (C) are displayed to indicate the structural similarity between proteins.


A
B
C

structure of the archaea-specific editing domain of the threonyl-tRNA synthetase (Dwivedi et al, 2005) (Figure 51C). Superposition of the alpha carbon atoms of DUE-B with each of the tRNA editing enzymes yielding root mean square deviation values in the range of 1.8-2.2 Å. Interestingly all obtained structures show the protein to be dimeric, consistent with gel filtration data obtained by our laboratory. A representative of both the monomeric subunit and the dimeric DUE-B are presented (Figure 49A).

Identified in the human DUE-B structure, but not reported in other published structures, is the presence of a Mg^{+2} ion in the proposed active site. The presence of the Mg^{+2} ion and the C-terminal extension of 60 amino acids may thus account for human DUE-B's unique function as a replication origin binding protein. The C-terminal ~60 amino acids unique to vertebrate homologs of DUE-B lacked interpretable electron density and are thus absent from the presented structure. In Figure 49B, the DUE-B dimer is oriented with the C-terminal end of the detectable structure at the bottom of the image, demonstrating that the unstructured C-terminal 60 amino acids of the individual monomers are projecting from a similar side of the molecule, and thus may coordinate together for DUE-B function.

DUE-B is a D-amino acid tRNA deacylase

Genetic and biochemical experiments demonstrated that the bacterial and yeast homologs of human DUE-B function as a D-amino acid tRNA deacylase (Lim et al, 2003; Soutourina et al, 1999; Soutourina et al, 2000; Soutourina et al, 2004). Although initially described as a D-tyrosyl-$tRNA^{Tyr}$ deacylase (Calendar and Berg, 1967; Soutourina et al, 1999), the enzyme has been shown to act on most tRNAs aminoacylated with a D-amino acid (Soutourina et al, 2000). The purpose of these proteins may be to function as a homochirality checkpoint in the process of protein synthesis by specifically recycling misaminoacylated D-amionacyl-tRNAs. A catalytic mechanism for D-tyrosy-$tRNA^{Tyr}$ deacylase had been proposed based on the crystal structure of the *Hemophilius influenzae* protein (Lim et al, 2003). In the model, Thr80 of the *H. influenzae* enzyme serves as the nucleophilic group that targets the carbonyl carbon of the aminoester linkage between the amino acid and tRNA. Interestingly, both this threonine and several other amino acids in the active site are conserved throughout evolution and are found in the human DUE-B sequence (Figure 52A). The probable catalytic residue in human DUE-B is Thr81, and is thus believed to function in identical fashion to that of the bacterial homologs (Figure 53A). Moreover, residues suggested

by Lim et al (Lim et al, 2003) as being involved in tRNA binding are conserved in the vertebrate DUE-B homologs as well, further arguing for a similar function for DUE-B.

Since Calendar and Berg had initially described the presence of D-tyrosyl $tRNA^{Tyr}$ deacylase activity in extracts from mammalian tissues (Calendar and Berg, 1967), and the only mammalian protein showing sequence or structural homology to the bacterial DTD1 proteins is DUE-B, we anticipated that human DUE-B also retained the ability to hydrolyze D-amino acids from aminoacylated-tRNAs. Using *E. coli* tRNAs and tRNA synthetases we incorporated [^{3}H]-D-aspartate onto tRNA. To test whether rDUE-B could then remove the D-aspartate from the tRNA, reactions were set up to contain the [^{3}H]-D-Asp-tRNA and either a wild-type, full-length rDUE-B (WT), a full-length rDUE-B where Thr81 was mutated to alanine (T81A), or a mutant rDUE-B lacking the C-terminal 62 amino acids (ΔCT) that resembles the smaller bacterial and yeast homologs. After a short incubation time the reactions were precipitated with ethanol and the supernatant analyzed for the presence of [^{3}H]-D-aspartate by liquid scintillation counting. As shown in Figure 52B, both the wild-type rDUE-B and the C-terminal truncation mutant enabled the release of [^{3}H]-D-aspartate from the aminoacylated tRNA. Noticeably deficient in activity was the T81A mutant, thus validating the proposed mechanism that D-aminoacyl-tRNA hydrolysis is Thr80/81-dependent.

The ATPase activity of DUE-B is Thr81-dependent

As previous data from our laboratory described ATPase activity in DUE-B (Casper et al, 2005), and the crystal structure we obtained showed similarities to the Rossman nucleotide binding fold and included a magnesium ion in the same location as the active site used for D-amino acid-tRNA hydrolysis, we were interested in whether the ATPase activity of DUE-B may function through a similar mechanism as that for the D-amino acid-tRNA deacylase activity (Figure 53). Divalent metal ions are often found in the active sites of enzymes that catalyze phophoryl transfer reactions (Steitz and Steitz, 1993; Doublie and Ellenberger, 1998; Yang et al, 2006), and may serve to either stabilize the developing charge on the γ phosphoryl oxygens during the transition state, to lower the pKa of the nucleophilic water molecule, or to selectively stabilize the substrate for in-line chemistry. The Oε1 of Gln-6 and the backbone carbonyls of Val-4 and Cys-28 appear to loosely coordinate the metal ion. Weak coordination of metal ions is also found in structures of several RNA polymerases, including those from poliovirus (Hansen et al, 1997; Ng et al,

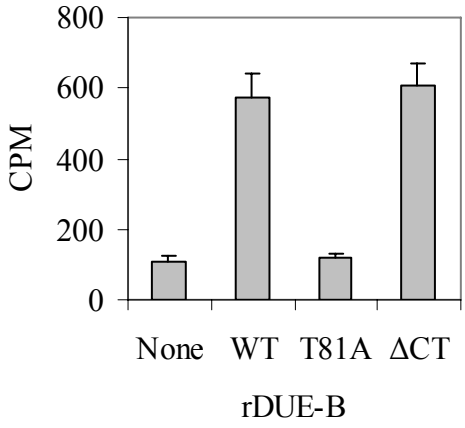
Figure 52. DUE-B possesses Thr81-dependent, D-amino acid tRNA deacylase activity like its structural homologs.

(A) Partial amino acid sequence of DUE-B, aligned at the proposed active site of the *E. coli* and *S. cerevisiae* D-tyrosine tRNA deacylases (asterisks represent identical amino acids). (B) rDUE-B^{Sf9} can hydrolyze D-aspartate from D-aspartate tRNA. E. coli tRNA aminoacylated with [^{3}H]-D-aspartate was treated with either wild-type (WT) DUE-B, a form of DUE-B with Thr81 mutated to alanine (T81A), or a form of DUE-B missing the C-terminal 62 amino acids (ΔCT). The relative amount of released [^{3}H]-D-aspartate was determined by liquid scintillation counting.

A

```
Human          -LCVSQFTL-
Budding Yeast  -LSVSQFTL-
E. coli        -LVVSQFTL-
               -* ******-
```

B



2002; Ng et al, 2004), rabbit hemorrhagic disease virus, and Norwalk virus. In these enzymes metal ions are only tightly bound in the active site in the presence of the substrate nucleotide.

To test whether the ATPase activity of DUE-B is Thr81-dependent, the ATPase activity of WT and T81A forms of rDUE-B were measured by thin layer chromatography (Figure 54A). Whereas WT DUE-B hydrolyzed ATP at a rate of ~80 fmol/min/pmol rDUE-B, the T81A mutant was significantly less active. Since the homologs of DUE-B lacking the C-terminal extension have not been reported to have ATPase activity, we were interested in whether the ΔCT form of DUE-B retained the ability of WT to hydrolyze ATP. Interestingly, ΔCT hydrolyzed ATP at approximately 3-4 times the rate of WT (Figure 54B), suggesting that the C-terminus of DUE-B may inhibit ATPase activity. Although it has not been demonstrated whether the bacterial and yeast homologs can hydrolyze ATP, their D-amino acid tRNA deacylase activities were shown to be stimulated when in the presence of magnesium (Soutourina et al, 1999).

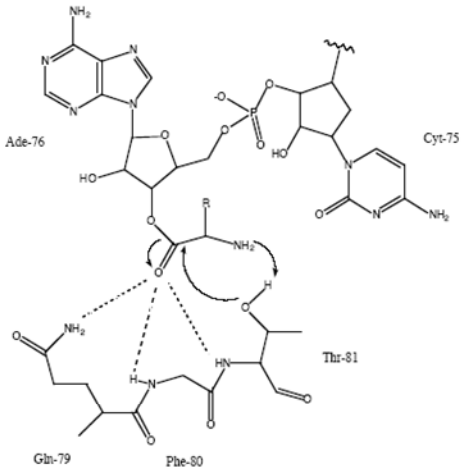
DUE-B as a multifunctional esterase

Interestingly, the PPM family of protein serine/threonine phosphatases similarly requires divalent metal ions for catalytic activity (Shi et al, 1998). Moreover, human DUE-B contains a seven motif arrangement of invariant amino acids essential for catalysis by PPM members, suggesting that DUE-B may be mixed function esterase. Because of DUE-B's phosphohydrolase activity on ATP, and its ability to bind tRNAs (Figure 52) and other oligonucleotides, we were interested in whether the esterase function of DUE-B may be extended to function as a ssDNA phosphatase. A 55 nt ssDNA containing either a 5'-phosphate and 3'-hydroxyl or both a 5'- and 3'-phosphate was exposed to DUE-B and then to the enzyme CircLigase to determine whether DUE-B had an ability to hydrolyze the phosphate off the 5' or 3' nucleotide. CircLigase activity requires a 5'-phosphate and 3'-hydroxyl group to circularize the ssDNA oligonucleotide, an event that can be easily visualized by SybrGold staining the products after electrophoresis on a denaturing polyacrylamide gel. Results showed that CircLigase acted only on the 5'-phosphate and 3'-hydroxyl substrate, and not the 5' and 3'-phosphorylated substrate, indicating that DUE-B is neither a 5'- nor 3'- ssDNA phosphatase (data not shown). Since these experiments gave negative results the conclusions to be drawn are limited, though it remains possible that DUE-B may act on different length DNA oligonucleotides or instead only on RNAs. Further experimentation will be needed to test this

Figure 53. Proposed catalytic mechanisms.

A comparison of the two chemical reactions carried out by DUE-B are displayed to highlight the mechanistic similarities. The D-aminoacyl-tRNA deacylase mechanism is displayed in (A), and the ATPase mechanism in (B).

A


Ade-76
Cyt-75
Thr-81
Gln-79
Phe-80

B

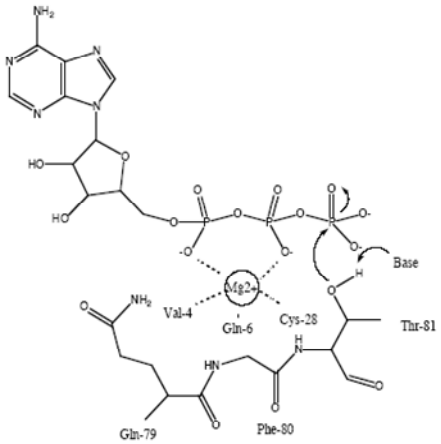
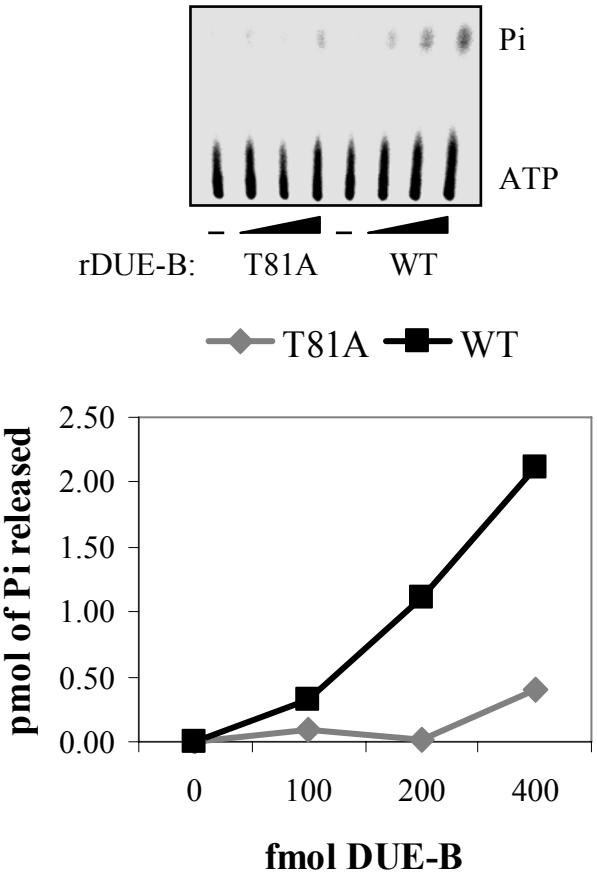

Base
Mg2+
Val-4
Gln-6
Cys-28
Thr-81
Gln-79
Phe-80

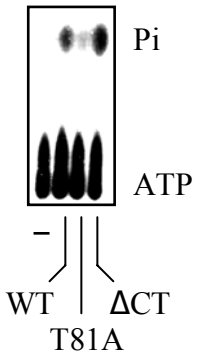
Figure 54. The ATPase activity of DUE-B is dependent on Thr81.

(A) Proposed mechanism for the hydrolysis of ATP by DUE-B. (B) The indicated recombinant forms of DUE-B were incubated in increasing amounts (100 ng, 200 ng, or 400 ng) in reactions with γ-^{32}P-ATP, then a portion applied to a PEI-cellulose TLC plate, developed, and scanned on a phosphorimager (top). A quantification of the amount of ATP hydrolyzed is presented (bottom). (C) A mutant form of DUE-B lacking the C-terminal 62 amino acids hydrolyzes ATP. Each of the indicated forms of DUE-B (400 ng) was incubated with γ-^{32}P-ATP and analyzed by TLC.

A


Pi
ATP
–
–
rDUE-B:
T81A
WT
T81A
WT
pmol of Pi released
2.50
2.00
1.50
1.00
0.50
0.00
0
100
200
400
fmol DUE-B

B


Pi
ATP
–
WT
ΔCT
T81A

hypothesis. It is also possible that DUE-B activities are restricted to D-aminoacyl tRNAs and ATP, or that DUE-B acts as a protein phosphatase, similar to the PPM class of phosphatases. Interestingly studies in *Xenopus* egg extracts demonstrated a requirement for phosphatase activity in the loading of Cdc45 onto chromatin (Chou et al, 2002; Lin et al, 1998). The similarity of DUE-B to the PPM family of protein phosphatases, and evidence in yeast showing that the DUE-B homolog DTD1 interacted with the PP2A-like protein phosphatase Sit4 (Gavin et al, 2002) together suggest the possibility that DUE-B may provide phosphatase activity at replication origins, either directly or through an interaction with vertebrate homologs of Sit4. Interestingly the Sit4 homolog in fission yeast (Ppe1) was shown to be required for cell cycle regulation (Bastians and Ponstingl, 1996; Bastians et al, 1997) and for equal chromosome segregation (Goshima et al, 2003), and the human homolog of Sit4 (PP6) rescues Sit4 temperature sensitive mutants in budding yeast (Bastians and Ponstingl, 1996).

The C-terminus of DUE-B is protease-sensitive and disordered

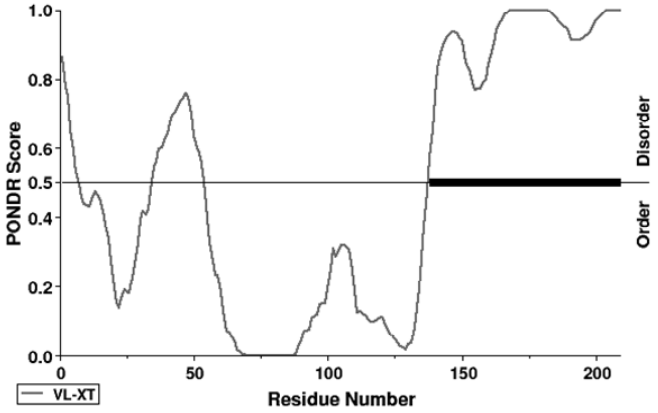
Because of the absence of interpretable electron density in the C-terminal region (amino acids 152-209) of the DUE-B crystal structure, and the finding that this domain is unique to vertebrate homologs of DUE-B, we were interested in exploring a potential novel function of the DUE-B C-terminus. Consistent with an inability to interpret the electron density of this domain, computational algorithms predicted the region to be disordered (Figure 55A). The PONDR algorithm can be used to predict the regions of proteins likely to be disordered proteins based on amino acid sequence, hydropathy, and protein flexibility (Iakouchev et al, 2001; Liu et al, 2006; Romero et al, 2004). The C-terminal domain of DUE-B (in bold, Figure 55B) is rich in both prolines and charged or hydrophilic amino acids (Figure 55C), consistent with the notion that the C-terminus may be a highly flexible domain.

Since disordered regions of proteins are typically more sensitive to digestion by proteases, we were interested in whether the DUE-B C-terminus might also be sensitive to protease digestion. Limited proteolytic digestion of rDUE-B with either trypsin or chymotrypsin rapidly gave rise to a domain of ~18 kDa in molecular weight, a size consistent with degradation of the C-terminus (Figure 56A). Interestingly this 18 kDa undigested product was shown to be largely resistant to any further degradation, and thus is indicated as the protease-resistant “core” of the protein. The protease resistance of this domain, which corresponds in size to the region crystallized in human DUE-B and its homologs, suggests that the

Figure 55. The C-terminus of DUE-B is predicted to be disordered.

(A) The DUE-B amino acid sequence was analyzed using the PONDR algorithm to predict the probability that regions of the protein are disordered. (B) Amino acid sequence of DUE-B with the C-terminal 62 amino acids of DUE-B in bold. An Asp-Pro bond sensitive to formic acid is underlined and in bold. (C) Amino acid composition of the C-terminus of DUE-B. The C-terminus is rich in both acidic and basic amino acids, as well as in prolines and hydrophylic amino acids.

A



B

MKAVVQRVTRASVTVGGEQISAIGRGICVLLGISLE
DTQKELEHMVRKILNLRVFEDESGKHWSKSVMDKQY
EILCVSQFTLQCVLKGNKPDFHLAMPTEQAEGFYNS
FLEQLRKTYRPELIKDGKFGAYMQVHIQNDGPVTIE
LES**PAPGTATSDPKQLSKLEKQQQRKEKTRAKGPSE**
SSKERNTPRKEDRSASSGAEGDVSSEREP

C

C-term 62 amino acid composition:

Pro: 6	Asp: 3	Ser: 10	Arg: 7
	Glu: 8	Thr: 4	Lys: 8

dimerization of DUE-B may be strong or stable. To test whether the proteases are targeting the C-terminus of DUE-B, samples of a trypsin digestion reaction were analyzed by western blot with anti-DUE-B antisera and an antibody targeting the 6xHis tag on the C-terminus of the recombinant protein. Loss of the anti-His reactivity corresponded with the emergence of the protease resistant core (Figure 56B). Similar results were obtained with V8 protease, an enzyme that hydrolyzes peptides on the carboxy-terminal side of glutamic acid residues (Figure 56B). Since formic acid has been shown to cleave proteins specifically between Asp-Pro bonds and DUE-B contains one target site at Asp156-Pro157 (indicated in Figure 55B), we employed formic acid digestion after trypsin proteolysis to confirm that the C-terminus of DUE-B is the protease-sensitive domain of DUE-B. As shown in Figure 56C, trypsin digestion of DUE-B yielded an ~18 kDa piece of DUE-B that was largely resistant to further digestion with formic acid. Interestingly, the full-length form of DUE-B was resistant to the high temperature (50°C) required for formic acid digestion, further arguing that the protein, or at least its N-terminal core domain, is a stable dimer. Together these data suggest that the employed proteases targeted amino acids in the DUE-B C-terminus after Pro157 and further argue that the C-terminus of DUE-B constitutes a protease sensitive domain.

Homodimerization of DUE-B is believed to be mediated by an extensive set of interactions that result in the formation of a continuous β-sheet structure between the two individual monomers. To determine whether the C-terminal 60 amino acids of DUE-B played any role in dimerization of the protein, extracts from HeLa cells transiently expressing a mutant of DUE-B cDNA lacking the C-terminal 62 amino acids and containing a 6xHis-tag were incubated with Ni-NTA agarose. The precipitated material included both the transfected ΔCT form of DUE-B as well as the full-length DUE-B endogenously expressed in HeLa cells (Figure 57A), arguing that the C-terminus is not required for dimerization. The ratio of ΔCT to endogenous DUE-B in the precipitate largely resembled that in the whole cell extract, suggesting that nearly all of the exogenously expressed DUE-B-ΔCT heterodimerizes with endogenous DUE-B.
Since these results indicated that heterodimerization occurs readily in vivo, the dynamics or stability of dimer formation was also studied in vitro. Gel filtration studies previously suggested rDUE-BSf9 was dimeric (Casper et al, 2005), consistent with crystallographic data. Addition of rDUE-BSf9 to either Xenopus egg extract or a HeLa nuclear extract showed no apparent heterodimerization in vitro (Figure

Figure 56. Protease digestion of DUE-B reveals a domain lacking the C-terminus.

(A) Recombinant DUE-B was incubated in reactions with either trypsin (1:200, w:w, 37°C) or chymotrypsin (1:500, w:w, 30°C) for the indicated length of time before stopping the reaction with SDS-PAGE sample buffer. Samples were then electrophoresed on a 12% SDS-PAGE gel and the gel was stained with silver. (B) Western blotting with anti-His antibody reveals that protease digestion cleaves peptides containing the 6xHis tag. DUE-B was digested with trypsin (1:20) as in (A) or with V8 protease (1:20, w/w) and samples were separated by SDS-PAGE and then analyzed by western blotting with anti-DUE-B and anti-His antibodies. (C) Formic acid digestion at the Asp-Pro bond does not significantly change the apparent size of the trypsin-resistant core. The location of the Asp-Pro bond is underlined in Figure 55B. Recombinant DUE-B was incubated in a reaction lacking or containing trypsin (1:100) for 2 hours before addition of formic acid to 70% and overnight (16 hours) incubation at 50°C. The samples were then lyophilized, neutralized, and separated on a 12% SDS-PAGE gel. Aliquots of the samples were analyzed by both silver staining and western blotting. Recombinant DUE-B maintained at 4°C is run in the first lane to show that the temperature incubations have no appreciable effect on the stability of DUE-B.

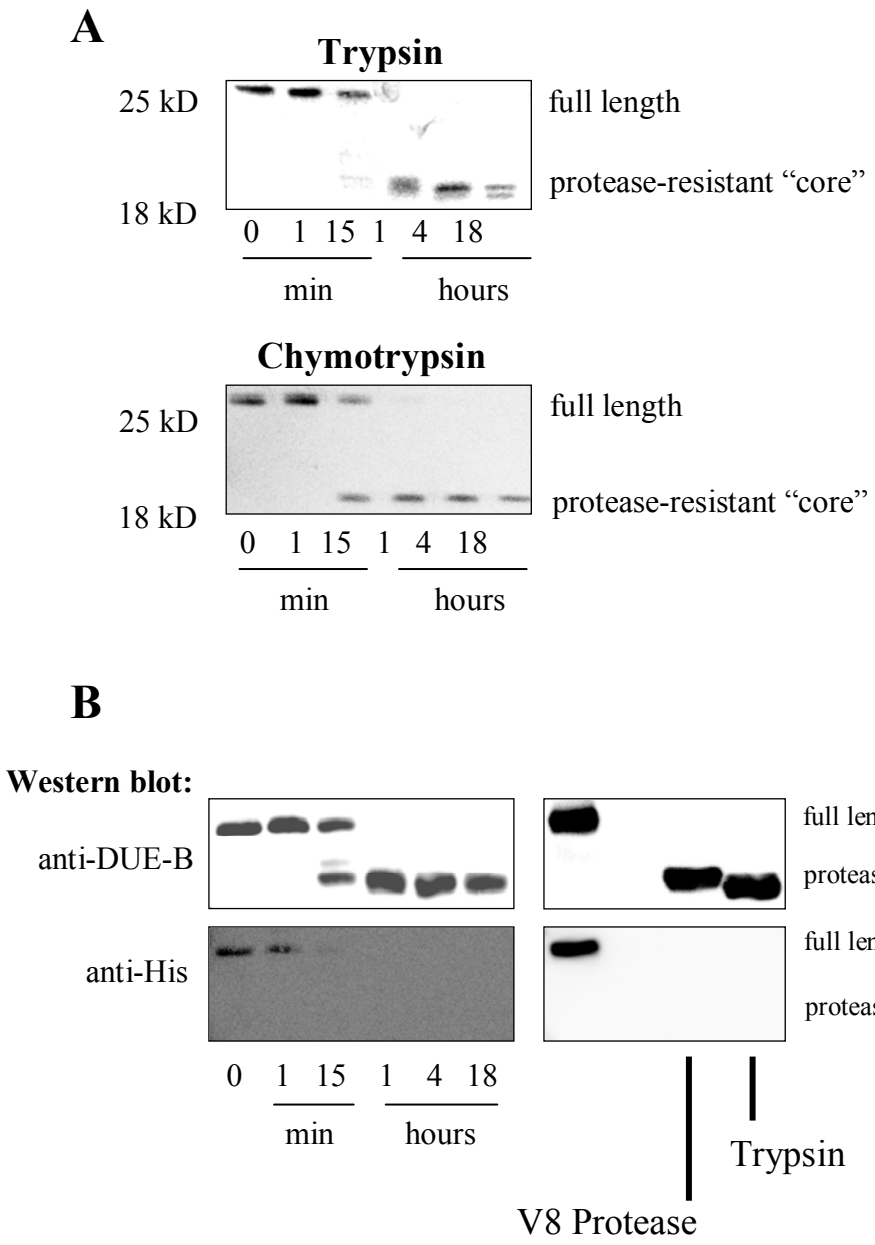
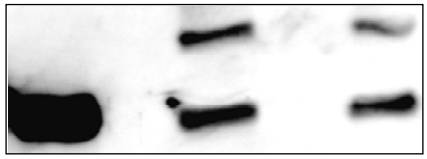

A
Trypsin
25 kD
18 kD
full length
protease-resistant "core"
0 1 15 1 4 18
min
hours
Chymotrypsin
25 kD
18 kD
full length
protease-resistant "core"
0 1 15 1 4 18
min
hours
B
Western blot:
anti-DUE-B
anti-His
full length
protease-resistant "core"
full length
protease-resistant "core"
0 1 15 1 4 18
min
hours
Trypsin
V8 Protease
C
Silver Stain
WB: DUE-B
4°C
— + — + Trypsin
— — + + Formic Acid
37°C/50°C

Figure 57. The N-terminal domain of DUE-B forms a stable, protease-resistant structure that does not require the C-terminus for dimerization.

(A) DUE-B lacking the C-terminus dimerizes with endogenous DUE-B in HeLa cells. A cDNA encoding a His-tagged DUE-B lacking the C-terminal 62 amino acids was transiently transfected and expressed in HeLa cells. Whole cell extracts (WCE) were then incubated with Ni-NTA agarose to pull down the His-tagged DUE-BΔCT, separated by SDS-PAGE, and analyzed by western blot with anti-DUE-B antibody. *In vitro* trypsin-digested recombinant DUE-B was run as a molecular weigh standard. (B) DUE-B does not readily heterodimerize *in vitro*. Recombinant DUE-B from baculovirus-infected insect cells was added to a Xenopus egg extract containing xDUE-B (left) or a HeLa nuclear extract (right). After incubation with Ni-NTA agarose, a portion of the bound (B) and unbound (U) fractions were analyzed by western blotting for DUE-B. (C) DUE-B dimerization is stable throughout the cell cycle *in vivo*. HeLa cells expressing a C-terminally His-tagged full-length DUE-B cDNA were arrested at the indicated phases of the cell cycle. Whole cell extracts were prepared and then incubated with Ni-NTA agarose. The material bound to the beads was analyzed by western blotting with anti-DUE-B antisera. (D) The N-terminal dimerization domain of DUE-B is resistant to proteolysis. Recombinant DUE-B (WT and ΔCT) incubated in reactions lacking or containing trypsin was analyzed by western blotting with anti-DUE-B antibody. (E) DUE-BΔCT is resistant to proteolysis. rDUE-BΔCT incubated in reactions lacking or containing trypsin was analyzed by western blotting with an anti-His antibody.

A

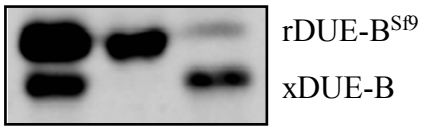


Endogenous HeLa DUE-B

DUE-BΔCterm

Trypsin-resistant core | WCE | Ni-NTA agarose Pull-down

B



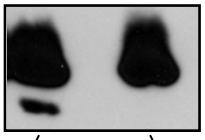
rDUE-B^{Sf9}

xDUE-B

B U

Egg extract + rDUE-B

Ni-NTA agarose pull-down



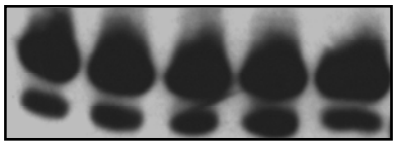
rDUE-B^{Sf9}

hDUE-B

HeLa NE + rDUE-B

Ni-NTA agarose pull-down

C



DUE-B-His$_6$

DUE-B

Asy | Mim (G1) | Aph (S) | HU (S) | Noc (M)

Ni-NTA agarose pull-down

D

anti-DUE-B western blot

Full-length

Core/ΔCT

− + − + Trypsin

WT ΔCT

E

anti-His western blot

rDUE-BΔCT

− + Trypsin

57B), suggesting that the exogenously added rDUE-B was a stable homodimer that does not readily dissociate to form heterodimers with xDUE-B and hDUE-B. The strong propensity for dimerization was similarly noted in the archaea-specific editing domain of threonyl-tRNA synthetase (ThrRS) (Dwivedi et al, 2005), the structure of which shows structural identity to human DUE-B and the bacterial DTD1 proteins. In many archaeal species this DUE-B N-terminus-like editing domain is fused to the ThrRS catalytic domain, which is itself also a homodimer. Furthermore, it is unlikely that the dimerization of DUE-B *in* vivo is regulated by cell cycle progression, since a 6xHis-tagged DUE-B cDNA expressed in HeLa cells shows heterodimerization with endogenous DUE-B throughout the cell cycle (Figure 57C).

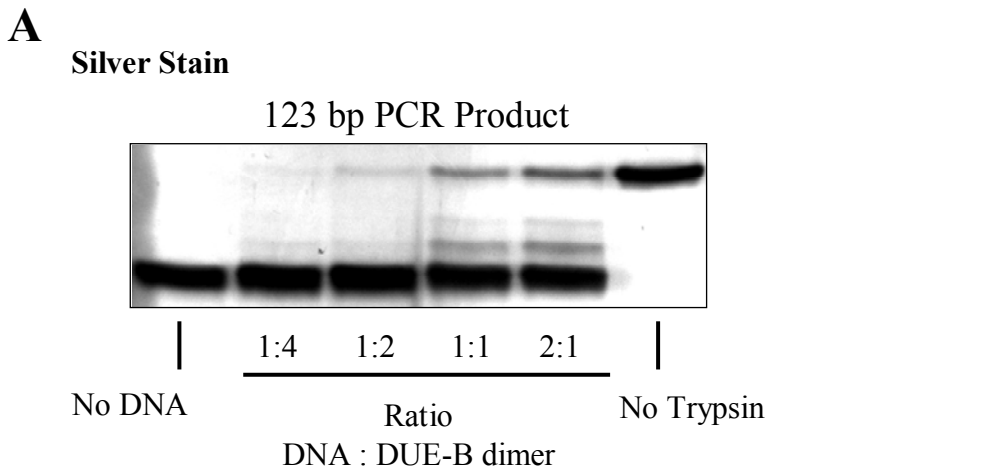
To demonstrate further that the N-terminal dimerization domain is stable and protease-resistant, the ΔCT mutant form of recombinant DUE-B from baculovirus-expressed insect cells was digested with trypsin. Whereas the full-length WT DUE-B is readily degraded to the "core" domain, the ΔCT form of DUE-B shows no detectable degradation, as determined by western blotting with anti-DUE-B (Figure 57D) or anti-His antibodies (Figure 57E).

Double-stranded DNA protects the DUE-B C-terminus from protease digestion

Disordered regions of proteins often adopt an ordered, protease-resistant structure upon interaction with ligands. For example the MCM helicase loader Cdc6 shows resistance to trypsin digestion when in the presence of ADP (Herbig et al, 1999). Since DUE-B was shown to bind DNA *in vitro* and chromatin *in vivo* (Figure 17), we tested whether the incubation of DUE-B with DNA allowed a stable structure to form in the DUE-B C-terminus. Limited proteolysis with trypsin was performed with rDUE-B after a short incubation with a variety of DNA substrates. As demonstrated in Figure 58A, a 123 bp PCR product containing the c-myc DUE protected the DUE-B C-terminus from degradation by trypsin. Annealed 54 nt DNA oligonucleotides from the c-myc DUE region similarly afforded DUE-B resistance to protease digestion, as indicated by both a protection of the full-length form of DUE-B as well as the emergence of intermediates visible in the digestion reaction time course, between the full-length and "core" sizes of DUE-B (Figure 58B). The 54 nt oligonucleotides in single-stranded, unannealed form did not provide the same level of protection as dsDNA (Figure 58C), indicating that DUE-B may preferentially bind dsDNA. The sequence of the DNA oligonucleotides may not be important, as poly-dIdC/poly-dIdC also provided the C-terminus protection from degradation (Figure 58D).

Figure 58. Double-stranded DNA protects DUE-B from protease digestion.

(A) The addition of a 123 bp dsDNA (generated by PCR) to trypsin digestion reactions protects the DUE-B C-terminus from degradation. Recombinant DUE-B was incubated with the indicated relative molar amount of DNA for 10 minutes at room temperature before the addition of trypsin (1:200, w/w) and incubation for 2 hours at 37°C. Reactions were stopped by addition of SDS-PAGE sample buffer, run on a 12% SDS-PAGE gel, and stained with silver. (B) A shorter, 54 bp DNA (annealed oligos) also protects the DUE-B C-terminus from protease degradation. Two complementary oligonucleotides were annealed, incubated with recombinant DUE-B (20:1, DNA:DUE-B) for 10 minutes at room temperature before addition of trypsin (1:400). The reactions were incubated at 37°C for the indicated period of time, then analyzed by SDS-PAGE and western blotting with anti-DUE-B antisera. (C) dsDNA protects the DUE-B from protease digestion better than ssDNA. Upper (U), lower (L), or annealed (U/L) 54 nt oligonucleotides were incubated with DUE-B for 10 minutes at room temperature before digestion with trypsin (1:200, w/w) for 2 hours at 37°C. Samples were analyzed by SDS-PAGE and silver staining. Note that the presence of DNA in these experiments facilitates the visualization of intermediates between the full-length form of DUE-B and a form lacking the C-terminal ~60 amino acids. (D) rDUE-B (200 ng) was incubated with 1.25 ng trypsin for 2 hours at 37°C in the presence or absence poly(dI-dC)·poly(dI-dC) (100 ng), and samples were analyzed by silver staining after SDS-PAGE.


A
Silver Stain
123 bp PCR Product
1:4
1:2
1:1
2:1
No DNA
Ratio
DNA : DUE-B dimer
No Trypsin
B
Western Blot
54 nt annealed oligos
Time
(min):
0
20
40
60
80
100
0
20
40
60
80
100
No DNA
+ dsDNA
C
Silver Stain
54 nt oligonucleotides
Full-length
Core/ΔCT
U
U/L
L
No DNA
No Trypsin
D
Silver Stain
Full-length
Core/ΔCT
—
+
poly-dIdC/dIdC

The DUE-B C-terminus is required for DNA binding *in vitro* but not chromatin binding *in vivo*

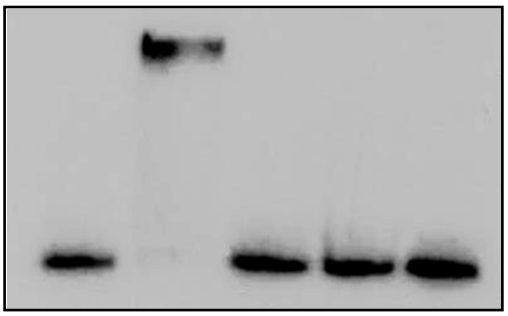
To confirm that the C-terminal domain of DUE-B binds DNA, electrophoretic mobility shift assays were performed with the full-length form of DUE-B and with forms of DUE-B lacking the C-terminus. Whereas full-length DUE-B is able to bind a 123 bp PCR product *in vitro* and form a shifted complex, elimination of the C-terminus by trypsin digestion or by use of rDUE-B-ΔCT prevented a stable interaction with dsDNA (Figure 59). These data argue that the ability of DUE-B to interact with DNA, and therefore with replication origins, may be through the C-terminal domain unique in vertebrate homologs of DUE-B.

Since the *in vitro* data argued that the interaction of DUE-B with DNA is likely through its C-terminal domain, we were interested in whether a similar phenomenon is observed *in vivo*. Preliminary experiments using cell fractionation and immunofluorescence indicated that DUE-B-ΔCT expressed in HeLa cells was found localized to the nucleus and on chromatin (data not shown). Since previous data showed that the endogenously expressed DUE-B in HeLa cells heterodimerizes *in vivo* with a form of DUE-B lacking the C-terminal 62 amino acids, we wanted to determine whether DUE-B-ΔCT homodimers were capable of localizing to chromatin. HeLa cells transiently expressing a DUE-B-ΔCT cDNA resistant to siRNA degradation were fractionated to yield soluble cytoplasmic, soluble nuclear, and chromatin-enriched fractions. As observed in Figure 60A, co-transfection with an siRNA targeting endogenous DUE-B for degradation did not appreciably alter the fraction of total DUE-B-ΔCT on chromatin as compared to cells co-transfected with a control siRNA. To be sure this was not an artifact of transient transfection, HeLa cells stably expressing siRNA resistant DUE-B-ΔCT were transfected with a control siRNA or with an siRNA targeting the endogenously expressed DUE-B. When chromatin was prepared from an equal number of cells treated with either siRNA, reduction in endogenous DUE-B levels had no effect on the

Figure 59. The C-terminus of DUE-B is required for DUE-B to bind DNA *in vitro*.

Recombinant forms of DUE-B (25 pmol, WT and ΔCT) were incubated in reactions lacking or containing trypsin (1:100 w/w) for 1 hour, then incubated at room temperature for 15 minutes with 25 fmol ^{32}P-labeled PCR product (123 bp). The samples were then loaded on a 4% native polyacrylamide gel (0.5X TBE) that had been pre-run for 30 minutes at 100 V. The samples were then electrophoresed for 50 minutes at 100V before drying the gel and autoradiographic analysis. Proper tryptic digestion of DUE-B was monitored by western blot.

EMSA

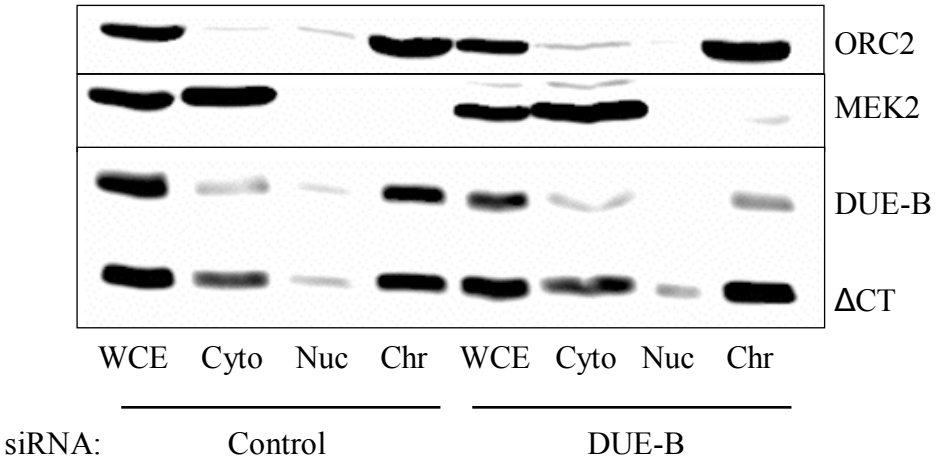


Trypsin:	−	−	+	−	+
rDUE-B:	−	WT		ΔCT	

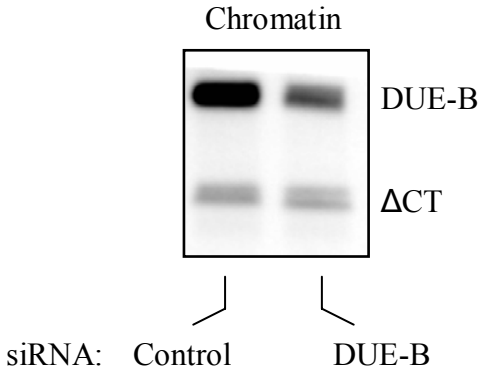
Figure 60. The C-terminus of DUE-B is not required for interaction with chromatin *in vivo*.

(A) HeLa cells transfected with 100 nM control or DUE-B siRNA were transfected again after 24 hours with both siRNA and a plasmid encoding the C-terminal truncation mutant of DUE-B. Six hours later the cells were re-plated, allowed to grow for an additional 20 hours, and then were fractionated to enrich for chromatin bound proteins. Aliquots of whole cell extract (WCE), a soluble cytoplasmic extract (cyto), a soluble nuclear extrac (nuc), and chromatin-enriched extract (Chr) were analyzed by SDS-PAGE and western blotting. Orc2 and MEK2 were analyzed as markers for the efficiency of the fractionation procedure. (B) HeLa cells or a HeLa cell line stably expressing DUE-BΔCT were transfected twice with control or DUE-B siRNA as above, but treated with 500 μM mimosine for 20 hours after the second transfection. After release from the arrest for 5 hours, cell were harvested and fractionated as above. The WCE and chromatin fractions were analyzed by western blotting.

A


ORC2
MEK2
DUE-B
ΔCT
WCE Cyto Nuc Chr WCE Cyto Nuc Chr
siRNA: Control DUE-B

B


Chromatin
DUE-B
ΔCT
siRNA: Control DUE-B

amount of DUE-B-ΔCT bound to chromatin (Figure 59B). Together these data argue that *in vivo* DUE-B may localize to chromatin through interactions with other chromatin-bound proteins, such as Dnmt1. Interestingly, although a GFP-tagged version of the yeast homolog of DUE-B (DTD1) was reported to be cytoplasmic (Huh et al, 2003), yeast DTD1 was found to interact with the evolutionarily conserved Rpb9 subunit of RNA polymerase II in a yeast two-hybrid assay (Ito et al, 2001). In vertebrate cells, it is possible that the DUE-B may be recruited to chromatin though an interaction with Rpb9 or other nuclear proteins, but may then through the C-terminal domain recognize a specific structural intermediate at the replication origin, such as unwinding DNA. Alternatively, the N-terminal tRNA-binding domain of DUE-B may recruit DUE-B to chromatin *in vivo* through an interaction with chromatin-associated oligonucleotides.

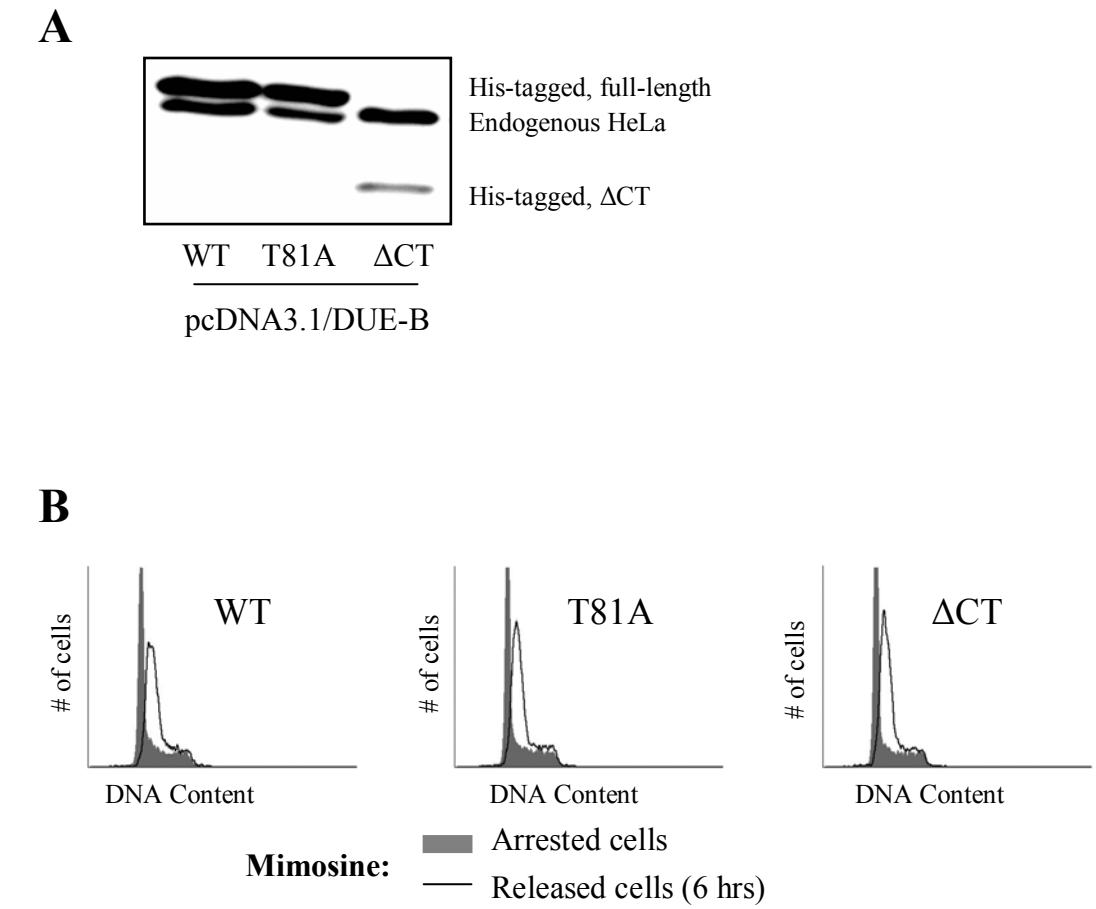
VII. Analysis of DUE-B mutants in human cells and *Xenopus* egg extracts

Since *in vitro* experiments established putative functions for both Thr81 and the C-terminus of DUE-B, we were interested in examining the phenotype *in vivo* of cells expressing these mutant forms of DUE-B. Stable cell lines expressing 6xHis-tagged full-length, wild-type DUE-B (WT), a Thr81Ala mutant (T81A), and a C-terminal truncation mutant (ΔCT) could all be successfully constructed using HeLa cells. The ΔCT form of DUE-B was expressed at a significantly reduced level compared to the other forms of DUE-B (Figure 61A). Transient expression of DUE-BΔCT was typically lower than that of the full-length forms of DUE-B (data not shown), suggesting that *in vivo* the C-terminus may help to stabilize the protein. Alternatively, the anti-DUE-B antisera may recognize epitopes on the C-terminus of DUE-B and provide a stronger detectable signal for these forms of DUE-B. Regardless, stable expression of the two mutant forms of DUE-B had no apparent effect on proliferation rate (data not shown) or on the ability to cycle through the cell cycle and enter S phase (Figure 61B). As all forms were constructed to be resistant to RNAi-mediated degradation by DUE-B siRNA#2, transfection with an siRNA targeting the endogenously expressed DUE-B reduced the endogenous protein levels while leaving the exogenously expressed forms of DUE-B intact (Figure 61C).

Although the goal of the construction of siRNA-resistant forms of DUE-B was to test the effect of mutant forms of DUE-B on S phase entry in the absence of endogenous DUE-B, a problem was encountered that suggested that the siRNA may have off-target effects that contribute to G1 arrest. Transient or stable overexpression of the wild-type, siRNA-resistant form of DUE-B was unable to rescue the effect of DUE-B siRNA#2 in experiments analyzing the rate of S phase entry after release from a mimosine-mediated late G1 phase arrest (data not shown). It is currently unclear precisely why this result was obtained, since the effect of the siRNA was shown to specifically act at the G1/S phase transition, and a separate siRNA targeting DUE-B had similar, albeit less potent, effects on cell cycle progression and proliferation. Additional experiments will be required to determine if there are conditions under which the siRNA resistant form of DUE-B can overcome effects of the siRNA. Alternatively additional siRNAs and corresponding resistant forms of DUE-B will need to be constructed and tested. It is also possible that placement of the 6xHis-tag on the C-terminus of these forms of DUE-B expressed in human cells renders the protein inactive *in vivo.*

Figure 61. Expression of DUE-B mutants in human cells has no effect on cell cycle progression or cell proliferation.

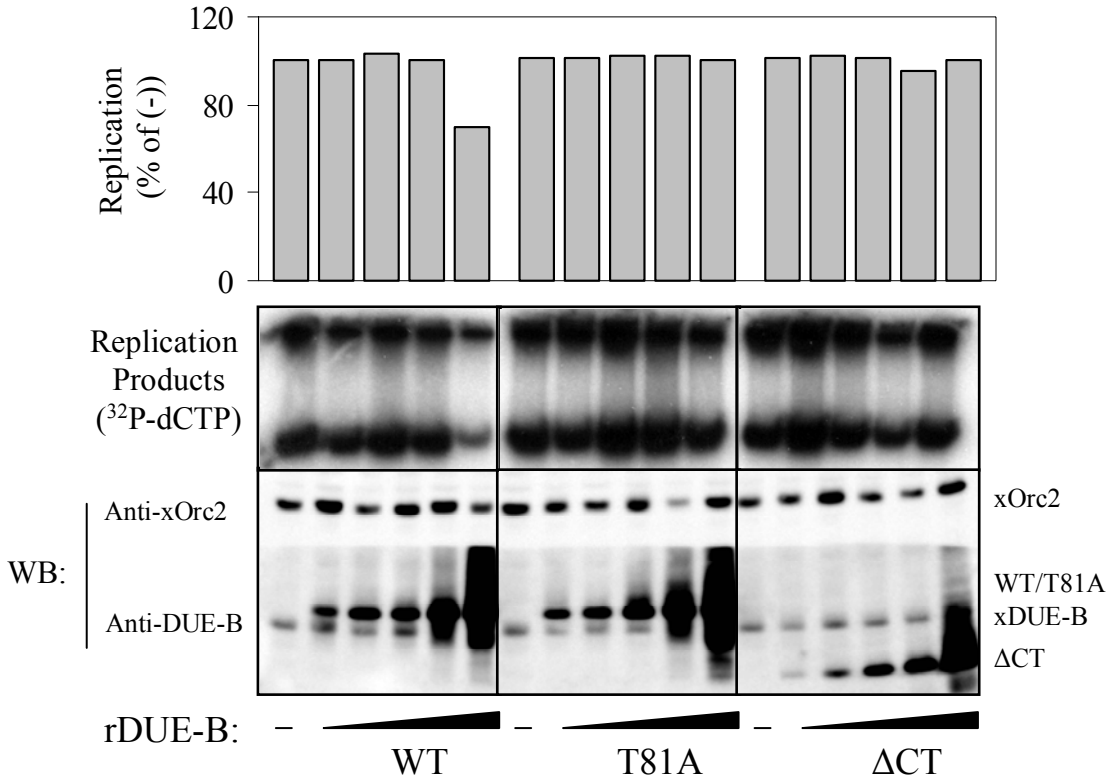
(A) Whole cell extracts of HeLa cell lines stably expressing siRNA-resistant forms of DUE-B. WT represents the normal, full-length human cDNA sequence. T81A contains an alanine in place of threonine at amino acid position 81. ΔCT represents a form of DUE-B lacking the C-terminal 62 amino acids normally found in human DUE-B. All contructs contains a 6xHis-tag at the C-terminus. (B) Cell lines stably expressing mutant forms of DUE-B (T81A and ΔCT) show no defect in entering S phase after release from an arrest in late G1 with mimosine. (C) The mutant forms of DUE-B are resistant to siRNA degradation. Whole cell extracts were prepared from HeLa cells and the clonal cell lines after two transfections with 100 nM of either control (C) or DUE-B (D) siRNA.


A
His-tagged, full-length
Endogenous HeLa
His-tagged, ΔCT
WT T81A ΔCT
pcDNA3.1/DUE-B
B
WT
T81A
ΔCT
# of cells
DNA Content
Mimosine:
Arrested cells
Released cells (6 hrs)
C
WT/T81A
Endogenous HeLa
ΔCT
siRNA: C D C D C D C D
Cell Line: HeLa WT T18A ΔCT
siRNA-resistant

To determine whether the mutant forms of recombinant DUE-B from baculovirus-infected insect cells altered the replication of sperm chromatin in the *Xenopus* egg extract system, the WT, T81A, and ΔCT forms of rDUE-B^{Sf9} were incubated in a low-speed *Xenopus* egg extract prior to the addition of DNA. Preliminary results suggested no apparent effect on DNA replication in this system, even at levels 20-fold higher than the endogenous xDUE-B in the extract (Figure 62). Although at the highest level of addition WT rDUE-B appeared to inhibit DNA replication, this result was not seen with the other forms of DUE-B. Moreover, as previous results had indicated that this form of rDUE-B inhibited sperm chromatin replication in the high-speed extract and nucleoplasmic systems, we anticipated that we would see a similar effect in the low-speed extract system. The data from this experiment and many similar assays (data not shown) suggest an apparent difference in the effect of rDUE-B^{Sf9} in the different types of *Xenopus* egg extract systems, and additional work will be needed to understand this difference in results. Moreover, whereas we could observe the binding of xDUE-B to sperm chromatin in low-speed egg extracts, we were unable to detect any binding of rDUE-B^{Sf9} to sperm chromatin under similar conditions. Whether this is due to a difference in phosphorylation or some other difference in activity is not yet clear. Since a previous experiment had indicated that rDUE-B^{Sf9} cannot functionally replace xDUE-B (Figure 44), addition of mutant forms of rDUE-B to xDUE-B depleted low-speed extracts was not attempted.

Figure 62. Addition of rDUE-B^{Sf9} mutants to Xenopus low-speed egg extracts has no effect on sperm chromatin replication.

rDUE-BSf9 (WT, T81A, ΔCT) was added to Xenopus low-speed egg extract at 20-, 10-, 5-, 2.5, and 1.25-fold levels compared to endogenous xDUE-B. Extracts containing or lacking rDUE-B were incubated for 30 minutes at room temperature before the addition of sperm chromatin to 2000/μl. Reactions were then incubated in the presence of α-^{32}P-dCTP for 60 minutes at room temperature, digested with proteinase K, and electrophoresed on a 0.8% agarose gel to detect replication products. The autoradiogram with quanititation is provided. Aliquots of the extracts were also analyzed by western blotting with anti-xOrc2 and anti-DUE-B antibodies, as indicated.


120
80
40
0
Replication
(% of (-))
Replication
Products
(^{32}P-dCTP)
WB:
Anti-xOrc2
Anti-DUE-B
xOrc2
WT/T81A
xDUE-B
ΔCT
rDUE-B:
–
–
–
WT
T81A
ΔCT

DISCUSSION

The data presented here suggest a role for two novel *trans*-acting factors that control the initiation of DNA replication in metazoans. Although histone acetylation has been recently recognized as a regulator of origin activity in budding yeast, and with roles in the activation of transcription in all eukaryotes including humans, it was not previously evident how it might be involved in the initiation of DNA replication in human cells. A second factor recognized as having a role at replication origins is the DNA unwinding element binding protein (DUE-B). Although DUE-B shows structural and functional homologs in bacterial and simple eukaryotes, vertebrate homologs of DUE-B contain a C-terminal DNA binding domain that may direct DUE-B to perform new functions. Together, this work has established histone acetylation and DUE-B as new factors that can control events at replication origins on metazoan chromosomes.

Histone acetylation as a replication initiation factor

Histone acetylation at replication initiation sites

We used a nascent strand abundance assay and chromatin immunoprecipitation to test whether a relationship existed between replication initiation and the level of histone acetylation at the human β-globin, lamin B2, and c-myc replication origin loci. Interestingly the early replicating lamin B2 and c-myc origin loci showed the level of histone H4 acetylation (AcH4) to be correlated with the abundance of short 1- to 2-kb nascent DNA produced at sites within these loci. The sites showing the highest level of acetylation and initiation site activity correspond to sites of known pre-RC formation and replication activity. These data are consistent with a model whereby histone acetylation contributes to or results from an open chromatin structure that facilitates replication initiation. Other factors presumably play a role in regulating replication initiation, since the β-globin origin locus did not show this same correlation between histone acetylation and initiation site activity. Nevertheless, the histone deacetylase inhibitor TSA induced a large increase in AcH4 at the β-globin replicator (STS-BG4) (Aladjem et al, 1998) and at other sites throughout the genome. A recent examination of the chicken β-globin locus suggested that even within the same locus different initiation sites may not show that same pattern of chromatin modifications (Prioleau et al 2003).

HDAC inhibition as a chromatin structure modifier

It is expected that modifying the acetylation level of histones will alter chromatin structure and change the accessibility of DNA for protein factors (Lowell and Pillus, 1998). We treated HeLa cells with the histone deacetylase inhibitor TSA and found the acetylation level of histones at the bulk chromatin level and at specific chromosomal sites within replication initiation zones to be increased. The observation that the extent of the increase varied among sites suggested a difference in HDAC or HAT localization at these sites, as previously proposed (Im et al, 2002; Johnson et al, 1998). Targeting of HDACs to the late-replication heterochromatin β-globin locus (McNairn,and Gilbert, 2003), or delayed recruitment of HATs may be responsible for the observation that the level of AcH4 increased only after longer periods of TSA treatment, a time at which a greater percentage of the cells are in late S phase.

Overall, the TSA-dependent increase in AcH4 varied inversely with basal level of AcH4, such that the highly acetylated c-myc locus showed a smaller relative increase in AcH4 compared to the β-globin locus. Within the early replicating c-myc and lamin B2 origin loci, sites flanking the primary initiation sites showed a larger relative increase in AcH4 after treatment with TSA, also supporting the notion that differential HDAC presence may restrict replication origin activation.

Decreased activity of the primary initiation sites at the c-myc and lamin B2 loci was observed after treatment with TSA, although no clear change in pattern was observed among flanking sites. A speculative model is that an increase in histone acetylation broadens the zone over which multiple initiations can occur. This increased acetylation may also lead to the activation of cryptic initiation sites, which was similarly suggested with the increased nascent strand abundance at genomic sites amplified with random sequence primers. Although there is very limited knowledge regarding pre-RC assembly in human cells, the HAT HBO1 interacts with the MCM helicase loading factor Cdt1 and is required for MCM loading at replication origins (Iizuka et al, 2006). Our observation that TSA induced an increase in MCMs on chromatin is consistent with this report. Together with indications that the multiple MCMs loaded per origin may allow for multiple potential initiation sites (Cvetic and Walter, 2005; Cvetic and Walter, 2006; Takahashi et al, 2005) within replication origin zone, these results suggest that a broadening of the zone over which replication can initiate may be due to an increase in the number of MCM complexes loaded at replication origins.

Replication origin activity and replication timing

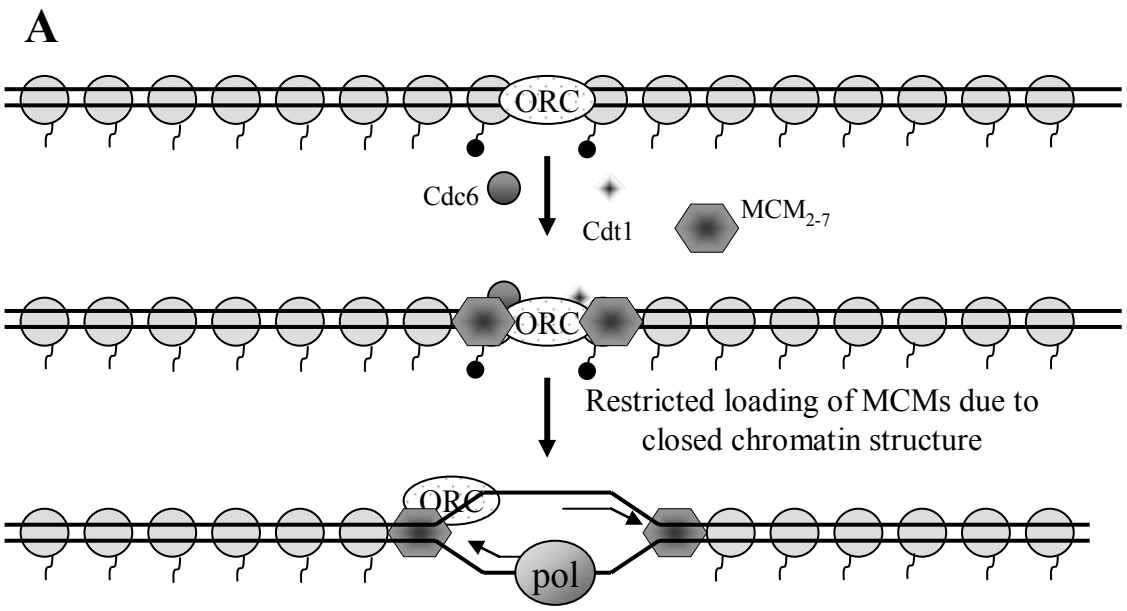
The observation that TSA caused replication to initiate from a site in the β-globin locus earlier in S phase may have contributed to the ability of TSA-treated cells to progress through S phase more rapidly after release a block in late G1 with mimosine, a compound that inhibits cells at a point prior to the establishment of active replication forks. Since this increased rate of progression through S phase could have been due to the faster progression of replication forks, cells were released from a block in early S phase with the polymerase inhibitor aphidicolin into medium containing TSA. Since we observed no measurable difference in S phase progression or completion in this situation, and aphidicolin synchronizes cells at a point ~2 hours after that of mimosine (Lalande, 1990), these results suggest that the primary step at which TSA affects replication is at the activation of replication complexes at replication origins and not by altering the rate of fork movement. Consistent with this interpretation, quantitation of total 1- to 2-kb nascent DNA from cells released from a mimosine block into medium lacking or containing TSA showed no significant difference (data not shown). Since fewer TSA-treated cells entered S phase (Figure 5), this indicated that more short nascent strands were produced per TSA-treated cell. Thus the initiation of DNA replication at new sites and at sites earlier in S phase probably contributes to the faster completion of S phase.

Model for histone acetylation at replication origins

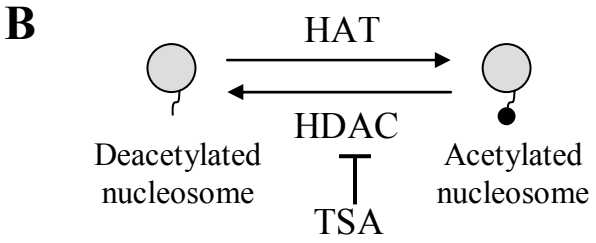
The data presented here are consistent with a model in which histone acetylation promotes the initiation of DNA synthesis by allowing access of replication initiation factors to the DNA template. Under normal circumstances, the binding of initiation factors to DNA may be restricted to those sites with a more open chromatin structure due to a higher basal level of acetylation (Figure 63A). Similarly, because the DNA flanking these sites are not bound to acetylated histones, the flanking chromatin is more closed and repressed, limiting the ability of ORC, Cdc6, and Cdt1 to load multiple MCM complexes at replication origins. This restriction in MCM loading would then give rise to the initiation of DNA synthesis over more limited region.

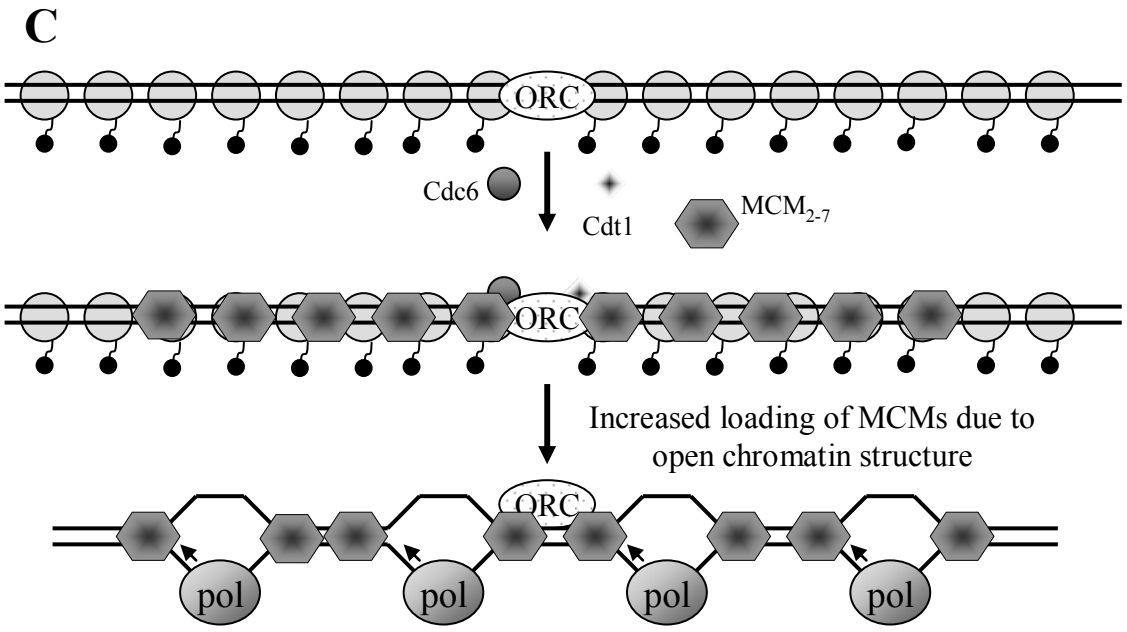
The acetylation state of histones is affected by the action of both HATs and HDACs that function through interactions with a variety of other nuclear proteins to control histone acetylation status. Through

Figure 63. Model for role of histone acetylation in the initiation of DNA replication in human cells. (A) At replication origins the formation of pre-RCs, and specifically the binding of MCMs, is restricted due to the closed or restricted chromatin structure at the origin (A), giving rise to a limited area from which DNA synthesis can begin. (B) The level of histone acetylation is regulated by HATs and HDACs, and chemical reagents can be used to inhibit HDACs. This inhibition would then favor the activity of HATs and yield hyperacetyled histones at the replication origin and subsequently a more open chromatin structure. (C) The increased level of acetylation allows an increase in the number of MCMs bound at each origin, such that the MCMs are distributed over a larger chromosomal domain, giving rise to the possibility of multiple potential sites for the initiation of DNA replication at the locus, with sites previously used with the highest frequency no longer being favored.



Restricted site of initiation





Increased number of potential sites of initiation
(each site used with reduced frequency)

the use of histone deacetylase inhibitors such as TSA, the balance of deacetylated versus acetylated histones can be shifted towards a hyperacetylated state (Figure 63B). The specific recruitment of HATs or HDACs to replication origins may also be a similar mechanism to control origin activity. On this note, additional data from our laboratory has shown that the recruitment of certain transcription factors to an inactive c-myc replicator construct converts it into an active replication origin (Ghosh et al, 2006). Interestingly, correlated with this recruitment is an increase in the level of histone H4 acetylation at the origin, further suggesting that increased acetylation promotes replication origin activity.

Our results examined the effects of TSA on replication origin activity, and suggest that by inhibiting HDACs and promoting the acetylation of histones both at known replication origins and globally throughout the genome, the pattern of replication origin activity can be altered (Figure 63C). It is likely that TSA affects both where along chromosomes pre-RCs can form, but TSA also may promote the assembly of multiple MCM complexes at replication origins (Figure 8B), and allow for easier activation of these pre-RCs, since at site in the β-globin locus replicated earlier after treatment with TSA. The increased levels of MCMs loaded per replication origin may give rise to a broader area over which DNA synthesis can initiate, with previously preferred sites used less frequently and new or inefficient sites used more often. Interestingly the activation of quiescent replication origins may be the opposite of what occurs during development and cell differentiation, where chromatin changes associated with cell type-specific gene expression serve to limit or restrict replication initiation to specific chromosomal sites.

DUE-B, a novel *trans*-acting replication origin binding protein

Identification of a DUE binding protein

The observation that replication origins in bacteria, yeast, eukaryotic viruses, and human cells often contain regions of helically unstable DNA suggests a role for these sequences in promoting the initiation of DNA replication. Since evidence from our laboratory had demonstrated such a DUE to be essential for replication initiation from the c-myc replication origin, we examined whether DUEs might also play a role in the recruitment of novel replication initiation factors to origins. Using a yeast one-hybrid assay, a protein termed the DNA unwinding element binding protein (DUE-B) was identified and later shown to bind both the c-myc and lamin B2 replication origins (Casper et al, 2005; Ghosh et al, 2006).

Furthermore, this binding is dependent on the presence of a functional DUE (Liu and Leffak, unpublished; Ghosh et al, 2006).

DUE-B is conserved throughout evolution, from bacteria to humans, but contains a unique extension of ~60 amino acids only in vertebrates. Antibodies raised against bacterially expressed recombinant human DUE-B protein show a high level of expression of DUE-B protein in both *Xenopus* egg extracts and human cells. Both human cell fractionation and immunofluorescence studies (Casper et al, 2005; Katrangi and Leffak, unpublished) demonstrate DUE-B to be localized largely to the nucleus and to chromatin throughout the cell cycle, similar to that of members of the origin recognition complex.

Consistent with the binding of DUE-B at replication origins, DUE-B was found to be a component of the DNA synthesome, a complex of >40 proteins most of which are found at the replication fork. DUE-B interacts specifically with the maintenance methyltransferase Dnmt1 in both *Xenopus* egg extracts and human cells. Dnmt1 is an essential protein that serves to maintain the methylation status of the genome during cell division, an event that is also correlated with formation of repressive chromatin structures. For example, the methyl-CpG protein MeCG recognizes methylated CpG residues and then recruits HDAC enzymes to promote the formation of repressive chromatin to inhibit transcription (Momparler, 2003). Thus DUE-B may similarly be involved in the regulation of chromatin structure.

The elevation of DUE-B protein levels in tumors suggests that increased DUE-B protein may contribute to the proliferative phenotype of cancer cells, an activity consistent with DUE-B binding a protein that binds replication origins. The observation that Daudi cells do not express any DUE-B mRNA or protein shows that DUE-B is not an absolutely essential protein, as was observed for its homologs in bacteria and yeast. It is possible that during the formation of this lymphoma these cells secondary mutations occurred such that cells no longer needed DUE-B. Attempts to reintroduce DUE-B into Daudi cells proved unsuccessful, though such experiments may be useful for determining a functional role for DUE-B in DNA replication and cell proliferation.

DUE-B promotes S phase entry in human cells

Two systems were employed in an attempt to elucidate the function of DUE-B at replication origins. In human cells DUE-B could be efficiently depleted from a variety of cultured cancer cell lines using RNA interference. Such reduction inhibited cell proliferation and induced cell death after 2-3 days.

The slowed proliferation appeared to be primarily through an arrest of cells in G1 phase, though the arrest was reversible since cells ultimately could enter S phase but with slower kinetics. By using several different cell lines, it was observed that the DUE-B knockdown phenotype was independent of p53 status. These results suggest that the absolute cellular level of DUE-B protein may be important for determining the rate at which cells activate their replication origins and begin to synthesizing DNA. By analyzing the chromatin binding of proteins involved in DNA replication initiation, cells with reduced DUE-B protein levels also displayed lower levels of the MCM helicase co-factor Cdc45 on chromatin. These results implicate a role for DUE-B in Cdc45 recruitment to replication origins and suggest further that a loss of DUE-B prevents the activation of replication origins. Knockdown of DUE-B expression via RNA interference also increased the protein level of the cyclin-dependent kinase inhibitor p21 and decreased the protein levels of three different cyclin-dependent kinases. The altered level of these proteins may also contribute to reduced Cdc45 loading onto chromatin.

Data suggesting a role for the replication origin binding protein DUE-B in S phase entry may be related to the recent proposition that mammalian cells contain a replication origin licensing checkpoint, based on a study linking ORC and Cdk2 activation in human cells (Machida and Dutta, 2005). Cell cycle checkpoints serve an important role in mammalian cells, preventing movement into the next phase of the cell cycle until all the events of the previous phase have been completed. For example, cells do not usually enter mitosis until the entire genomic DNA content has been replicated, presumably to prevent chromosome loss. Similarly cells exposed to DNA damaging agents stop replicating DNA via the S phase checkpoint, thus preventing the accumulation of mutations (Gottifredi and Prives, 2005). Interestingly a recent report showed that acute reduction of Orc2 levels induced the expression of the CDK inhibitors p21 and p27, resulting in a decrease in cyclin E-Cdk2 kinase activity and an arrest in late G1 phase of the cell cycle (Machida et al, 2005). Furthermore, cells hypomorphic for Orc2 shows defects in pre-RC formation on chromatin and a reduced ability to activate Cdk2 (Teer et al, 2006). One of the mechanisms to prevent re-replication of genomic DNA is the restriction of pre-RC formation to periods when CDK activity is low, such as occurs during late mitosis and G1 phase (Dahmann et al, 1995; Detweiler and Li, 1998; Hua et al, 1997). Thus the premature activation of Cdk2 before a sufficient number of origins have been licensed could push the cells irreversibly into S phase without the ability to form more pre-RCs and replicate the

entire length of chromosomes. In yeast, this type of replication from too few origins led to chromosomal damage (Lemoine et al, 2005; Lengronne and Schwob, 2002; Tanaka and Diffley, 2002). Thus an ORC-dependent activation of Cdk2 could serve as a checkpoint for origin formation and activation in mammalian cells, only allowing Cdk2 activation after sufficient pre-RC formation. The observation that human cells depleted of DUE-B arrest in G1 with elevated p21 levels and reduced CDK levels may similarly suggest a role for DUE-B in linking origin licensing to cell cycle progression to maintain genome stability in human cells.

xDUE-B is required for DNA replication in *Xenopus* egg extracts

To determine whether DUE-B has a role in DNA replication using a different experimental system, xDUE-B was depleted from *Xenopus* high-speed or low-speed egg extracts using anti-human DUE-B antisera. When used in sperm chromatin replication assays, these extracts yielded significantly reduced levels of DNA replication, similar to that observed in xOrc2 depleted extracts. This inhibition was sensitive to the degree of depletion of xDUE-B, and quantitative depletion studies showed that >99% of xDUE-B needed to be removed from the extract to show a defect in DNA replication. To show that this replication inhibition was specifically due to the loss of DUE-B, recombinant DUE-B purified from HeLa cells (rDUE-B^{HeLa}) was added back to the xDUE-B-depleted extract and sperm chromatin replication assays performed. When added back in excess to the amount of xDUE-B normally present in the extract, rDUE-B^{HeLa} could successfully restore replication competence to the extract. These results argue that DUE-B is required for chromosomal DNA replication in the embryonic *Xenopus* egg extract system.

Since previous results from our laboratory had shown that recombinant DUE-B from baculovirus-infected insect cells (rDUE-B^{Sf9}) inhibited sperm chromatin replication in either the high-speed egg extract or NPE replication systems, we were curious whether rDUE-B^{Sf9} could complement xDUE-B in egg extracts. A preliminary experiment suggested that it could not restore replication to xDUE-B-depleted egg extracts. Since we observed that rDUE-B^{Sf9} but not xDUE-B became phosphorylated when incubated in replication-competent egg extracts, we were interested in whether there might be a difference in post-translational modification between rDUE-B^{Sf9} and rDUE-B^{HeLa}. Phosphoserine-western blot and mass spectrometry analyses suggested the presence of additional phosphoserine residues in rDUE-B^{HeLa} that were lacking in rDUE-B^{Sf9}. These results are consistent with the apparent difference in activities between

the two forms of DUE-B in *Xenopus* egg extracts. Further work will be necessary to determine precisely how the phosphorylation of DUE-B regulates its activity in *Xenopus* egg extracts.

DUE-B is phosphorylated by CK2 *in vitro* and likely *in vivo*

Mass spectrometric analysis suggested that a C-terminal peptide of rDUE-B^{HeLa} contained three phosphorylated residues. Although this peptide contains 5 serines, three match the consensus sequence for casein kinase II (CK2). When rDUE-B^{Sf9} was added to HeLa nuclear extracts containing or lacking the CK2 inhibitor TBB, it became phosphorylated in a CK2-dependent manner. *In vitro* recombinant CK2 directly phosphorylated DUE-B within its C-terminal domain, thus suggesting a role for CK2 in the phosphorylation of DUE-B both *in vitro* and *in vivo.* Although CK2 has several hundred cellular protein targets (Olsten and Litchfield, 2004; Olsten et al, 2005), CK2 has emerged as an important kinase regulating a variety of processes in DNA replication, repair, and checkpoint signaling (Cheung et al, 2005; Koch et al, 2004; Loizou et al, 2004; Morales and Carpenter, 2004; Yamane and Kinsella, 2005a; Yamane and Kinsella, 2005b). Interestingly, a recent report demonstrated that the major kinase phosphorylating Orc2 in *Drosophila* embryonic extracts was CK2, resulting in altered binding of ORC to DNA (Remus et al, 2005). It remains to be determined just how CK2 phosphorylation regulates DUE-B activity, but the apparent difference in effects of rDUE-B^{HeLa} and rDUE-B^{Sf9} in xDUE-B depleted egg extracts correlates with the presence of this peptide containing CK2 target sites. CK2 has been reported to promote localization of a number of proteins to the nucleus (Pinna, 1990; Rihs et al, 1991) and thus the inability to detect rDUE-B^{Sf9} on chromatin (data not shown) and its inability to restore replication to xDUE-B depleted egg extracts (Figure 44) might be due to a lack of phosphorylation by CK2 in the C-terminal, DNA-binding region of rDUE-B^{Sf9}.

Differences in activity between rDUE-B^{Sf9} and rDUE-B^{HeLa} or HeLa/*Xenopus* DUE-B

The results presented here highlight apparent differences in DUE-B function depending on the source of the protein employed. Whereas recombinant DUE-B expressed in either bacteria or baculovirus inhibited sperm chromatin replication when preincubated with DNA in a high-speed *Xenopus* egg extract

Table 6. List of differences between rDUE-B^{Sf9} and rDUE-B^{HeLa}/HeLa/Xenopus DUE-B

A summary of the differences observed among the forms of DUE-B are displayed.

Assay	rDUE-B^{Sf9}	rDUE-B^{HeLa}/HeLa/*Xenopus* DUE-B
Gel Filtration (purified protein)	Elution consistent with dimeric structure	N/A
Gel Filtration (*Xenopus* egg extract or HeLa nuclear extract)	Forms high molecular weight complex	Elution consistent with dimeric structure
Mass spectrometry (MALDI-TOF)	No unique peptides	Unique C-terminal peptide mass consistent with presence of three phosphoserine residues
Anti-phosphoserine antibody reactivity	+	++
Phosphorylated by CK2 *in vitro*?	+++	+
Phosphorylated upon incubation in *Xenopus* egg extract?	Yes	Not detected
Functionally replace xDUE-B in *Xenopus* egg extracts?	No	Yes

prior to the formation of nuclei, depletion of xDUE-B from either low-speed or high-speed *Xenopus* egg extract also resulted in defective chromosomal replication (Casper et al, 2005). The data presented here argue that the dissimilar activities may be due to differences in the level of phosphorylation between the forms of DUE-B. These results are summarized in Table 6, and are consistent with earlier gel filtration analyses showing an apparent difference in complex formation between rDUE-B^{Sf9} and DUE-B expressed endogenously in *Xenopus* egg extracts or HeLa nuclei. Whether the phosphorylation of DUE-B by CK2 alters DUE-B activity *in vivo* is currently unknown, and clearly additional work is needed to test this hypothesis.

Since recombinant DUE-B expressed in both bacteria and baculovirus inhibited sperm chromatin replication in *Xenopus* egg extracts under specific experimental conditions, it is likely that these two forms of DUE-B share something in common. Whereas baculovirus expressed rDUE-B (rDUE-B^{Sf9}) was shown by gel filtration to be dimeric, a 6xHis- and Myc-tagged DUE-B expressed and purified from *E. coli* eluted by gel filtration chromatography with a monomeric molecular weight (Casper et al, 2005). Since bacteria express a protein with a homologous structure (D-aminoacyl-tRNA deacylase), it is currently unclear why the bacterially expressed protein was not dimeric, though it could be due to the addition of the Myc-tag unique to the bacterial expression vector. However, since mass spectrometric analysis suggested phosphorylation on the C-terminus of rDUE-B^{HeLa} but not on rDUE-B^{Sf9}, and recombinant proteins expressed in bacteria are widely recognized as lacking the normal posttranslational modifications found in eukaryotic cells, it is possible that a dephosphorylated form of the C-terminus binds DNA and replication origins in an replication-inhibitory manner. These results are consistent with a model in which the phosphorylation of the C-terminal DNA-binding domain of these forms of recombinant DUE-B may be responsible for the function of DUE-B in DNA replication. Experiments are underway to test this model. It is also possible that the phosphorylation of DUE-B regulates its interaction with an additional, as yet unknown, protein factor required for the initiation of DNA replication. In this scenario, the dephosphorylated form of DUE-B may be unable to bind to or recruit this factor to replication origins.

The crystal structure of DUE-B

The marked sequence similarity between the N-terminal ~150 amino acids of DUE-B and that of D-tyrosyl-$tRNA^{Tyr}$ deacylases (DTD1) from yeast and bacteria imply similarities in structure and function

of these homologs. In collaboration with Dr. Satish Nair, we obtained an x-ray structure of this domain to 2.0 Å resolution. Comparison to the previously reported structures of the *E. coli* and *H.influenzae* DTD1 enzymes (Ferri-Fioni et al, 2001; Lim et al, 2003) showed nearly identical structures (data not shown). More recently, an editing domain on archaeal threonine-tRNA synthetase (Beebe et al, 2004; Korencic et al, 2004) was first computationally predicted (Rigden, 2004) and then experimentally demonstrated to be strikingly similar to the reported bacterial DTD1 enzymes (Dwivedi et al, 2005). Conserved within a putative active site of the bacterial DTD1 proteins and DUE-B is a threonine residue (Thr80/81) proposed to be the amino acid catalyzing hydrolysis of the aminoester linkage between D-amino acids and the tRNA (Lim et al, 2003; Yang et al, 2003). The C-terminal 60 amino acids of DUE-B, which are unique to vertebrate homologs of DUE-B and are predicted to be unstructured, were uninterpretable in the obtained structure. As discussed below, preliminary evidence suggests this domain of DUE-B may serve to bind DNA.

DUE-B is a D-amino acid tRNA deacylase

The marked structural similarity between the bacterial DTD1 enzymes and DUE-B suggested a similarity in function. To test whether DUE-B possessed D-amino acid tRNA deacylase activity, *E. coli* tRNA was aminoacylated with D-aspartate and then exposed to either wild-type DUE-B or mutant forms of DUE-B. Consistent with a report by Calendar and Berg (Calendar and Berg, 1967) that demonstrated mammalian tissues to contain D-tyrosyl tRNA deacylase activity, recombinant DUE-B containing or lacking the C-terminal 60 amino acids could hydrolyze D-aspartate off of the aminoacylated tRNA. In contrast, a mutant rDUE-B with Thr81 replaced with Ala was unable to perform this function, thus validating the catalytic mechanism proposed by Herzberg and colleagues (Lim et al, 2003).

The presence of a D-amino acid tRNA deacylase in all living organisms suggests a biological and physiological need for this enzyme for viability that has been conserved through evolution. The enzyme may have arisen early in the evolution of life to select for the proper chirality of amino acids being incorporated into proteins, although RNA-directed fixation of homochirality of amino acids in proteins has also been suggested and supported by experimental studies (Tamura and Schimmel, 2004). The primordial aminoacyl-tRNA synthetases (aaRSs) may have played a critical role in enforcing this homochirality during translation, since aaRSs must deal with difficult potential substrate recognition problems, and thus many

have evolved special modules for editing purposes. Nevertheless, it is well recognized that aminoacyl-tRNA synthetases can esterify tRNAs with D-amino acids (Calendar and Berg, 1967), although with varying efficiencies.

Deletion of the DUE-B homolog in yeast and bacteria is toxic to organisms exposed to D-amino acids (Soutourina et al, 2000), due to the accumulation of high amounts of D-aminoacylated-tRNAs and a starvation for L-aminoacylated-tRNAs (Soutourina et al, 2004). Overexpression of DTD1 relieved this starvation by increasing the amount of cellular L-aminoacyl-tRNA available for translation (Soutourina et al, 2004). Some species, such as *Bacillus subtilis,* which lack a start codon for the DTD1 gene, shows extreme susceptibility to growth inhibition by D-tyrosine (Champney and Jensen, 1969; Champney and Jensen, 1970). It is currently unknown whether this enzyme is required in vertebrates or humans to prevent the misincorporation of D-amino acids into proteins, since there are a variety of oxidases in human cells that act specifically on D-amino acids to convert them to the L stereoisomers. For example, D-aspartate oxidase is known to selectively metabolize D-aspartate *in vitro,* and mice lacking this enzyme show increased amounts of D-aspartic acid and NMDA in all tissues examined (Errico et al, 2006). However D-amino acids also have important roles in human physiology. For example, significant concentrations of D-aspartic acid and D-serine are present in the central nervous system and endocrine glands in mammals (Hashimoto et al, 1993a; Hashimoto and Oka, 1997), particularly in embryos (D'Aniello et al, 2000; Furuchi and Homma, 2005; Wang et al, 2000). In the rat hypothalamus, free D-aspartate localizes subcellularly to the heterochromatin in the nucleoli (Wang et al, 2002), suggesting a physiological role in nuclear function. In cultured rat Leydig cells, D-aspartate promotes the synthesis of testosterone (Nagata et al, 1999). High levels of D-serine have been found in the mammalian brain where it activates glutamate/NMDA receptors by interacting with the glycine site of the receptor (Hashimoto et al, 1993b; Hashimoto and Oka, 1997). Together these results suggest physiological functions for free D-amino acids in mammalian cells and suggest the possibility that mechanisms to prevent their incorporation into proteins are present in human cells.

It should also be noted that it is quite common to find aminoacyl-tRNA synthetases (aaRSs) or aaRS-like proteins with functions outside that of protein synthesis (Lee et al, 2004; Park et al, 2005; Schimmel and Ribas De Pouplana, 2000), including DNA replication and other nuclear processes. For

example the *E. coli* alanine-tRNA synthetase repressed expression of its own gene by binding specifically to a palindromic sequence that flanks the gene's transcription start site (Putney and Schimmel, 1981). In wheat embryos and HeLa cells the tryptophanyl-tRNA synthetase co-purified with and stimulated DNA polymerase alpha activity (Castroviejo et al, 1982; Rapaport et al, 1981). Even more striking, the accessory subunit B of mitochrondrial polymerase γ from several metazoan species shows structural homology to bacterial glycyl-tRNA synthetases (Carrodeguas et al, 1999; Carrodeguas et al, 2001; Fan et al, 1999). Furthermore, deletion of conserved protein sequences from the polγB subunit that are required for aminoacyl-tRNA activity demonstrated these regions to be important for interaction with polγA and for activity as a processivity factor (Carrodeguas and Bogenhagen, 2000).

In mammalian cells, nine aminoacyl-tRNA synthetases form a stable complex with several non-synthetase accessory proteins. One of these factors, p18/AIMP3 was recently demonstrated to be induced and translocated to the nucleus in response to DNA damage, where it was involved in and required for the activation of p53 and the DNA damage signaling kinase ATM (Abraham, 2005; Park et al, 2005). Similarly, p38/AIMP2 translocates to the nucleus after stimulation with transforming growth factor-β (TGF-β), where it leads to the ubiquitination and downregulation of the FUSE-binding protein (FBP), repressing c-myc expression (Kim et al, 2003). Interestingly, the FBP binds to the same region of DNA upstream of the c-myc gene as does DUE-B.

Together, these data argue that aminoacyl-tRNA synthetases and their associated proteins, as well as proteins structurally related to tRNA metabolizing enzymes, have significant roles outside protein synthesis that may regulate additional processes controlling cell proliferation and genome stability. Thus DUE-B may represent another homolog of a tRNA metabolizing enzyme that has evolved additional roles in DNA replication and cell proliferation.

DUE-B is an ATPase

Since DUE-B was previously shown to possess the ability to hydrolyze ATP, we were curious whether it may act by a similar catalytic mechanism, particularly because of the presence of a Mg^{+2} ion in the DUE-B active site. Whereas wild-type DUE-B could hydrolyze ATP at ~80 fmol/min/pmol protein, the Thr81Ala mutant form of DUE-B displayed significantly reduced activity. Interestingly, the ΔCT form of DUE-B that lacks the C-terminal 62 amino acids of DUE-B could apparently hydrolyze ATP at a faster rate

than wild-type, suggesting that the C-terminus may actually inhibit enzyme activity. It is currently unclear as to the significance of this activity, since overexpression of DUE-BΔCT in either human cells or *Xenopus* egg extracts had no detectable effect on DNA replication.

Data demonstrating multiple activities for the DUE-B protein suggested that DUE-B may be a multifunctional esterase. Although preliminary experiments suggested DUE-B could not hydrolyze the phosphate off the 5'- or 3'-terminal end of a 54 nt ssDNA, it remains to be determined whether there are other substrates on which DUE-B can act. We are also interested in the possibility of DUE-B being a Ser/Thr phosphatase, based on the conserved presence of a seven motif arrangement of invariant amino acids found in the PPM family of protein phosphatases. This family of phosphatases requires both a divalent cation and the seven invariant amino acids for activity, and future work will test the hypothesis that DUE-B shares this activity.

The DUE-B C-terminus

The inability to decipher electron density in the C-terminal 58 amino acids of the DUE-B crystal structure was consistent with computational predictions that the sequence is flexible or disordered. Such regions of proteins are often very sensitive to digestion *in vitro* with proteases, and our studies confirm that the C-terminus is readily degraded by a trypsin, chymotrypsin, and V8 protease. Since DUE-B binds DNA *in vitro* and *in vivo*, and the C-terminus is rich in basic amino acids that might be contribute to this binding, we tested whether the proteolytic digestion of the C-terminus of DUE-B was protected after incubation with a variety of DNA substrates. Indeed we found that whereas a variety of double-stranded DNA substrates readily prevented degradation of the C-terminus, single-stranded DNA provided little or no protection. These results suggest that the DUE-B C-terminus adopts an ordered confirmation when in the presence of double-stranded DNA. Interestingly, the FUSE-binding protein (FBP), a regulator of c-myc transcription, binds the same region of DNA (DUE/FUSE) as DUE-B, but instead preferentially binds to single-stranded DNA. Thus DUE-B and FBP may compete *in vivo* for binding to the DUE/FUSE region of the c-myc promote, thus controlling replication and/or transcription at this locus.

To confirm that the C-terminus of DUE-B is required for DUE-B to bind DNA, recombinant DUE-BΔCT or the DUE-B proteolytic resistant core were examined for complex formation with dsDNA by electrophoretic mobility shift assay. The results indicated a requirement for the C-terminus in this binding,

and further work will be necessary to determine if the C-terminus alone is sufficient for DNA binding. As previous DNA binding assays had shown specific binding of rDUE-B to DNA only when incubated with a HeLa nuclear extract, it remains to be determined whether rDUE-B binding to DNA is dependent on sequence or a particular structural conformation of the replication origin. Moreover, since rDUE-B becomes phosphorylated in a CK2-dependent manner in HeLa nuclear extracts, the increased binding specificity of rDUE-B towards DNA may be a result of phosphorylation by CK2.

Since the yeast homolog of DUE-B (DTD1) lacks the C-terminal extension and was reported to be cytoplasmic when fused to GFP (Ghaemmaghami et al, 2003; Huh et al, 2003) we were curious whether the localization of DUE-B to the nucleus and to chromatin was dependent on the presence of the C-terminus. Interestingly, we observed that *in vivo* DUE-B localizes on chromatin even in the absence of a C-terminal extension. Immunofluorescence studies have confirmed that the ΔCT form of DUE-B is recruited to nuclear foci, even after knockdown of endogenous DUE-B protein levels in HeLa cells (Katrangi and Leffak, unpublished). Thus DUE-B recruitment to DNA replication origins *in vivo* may be mediated through an interaction with other nuclear proteins. Although Dnmt1 could be a candidate protein for this role based on previous evidence that it interacts with DUE-B in human cells and *Xenopus* egg extracts, Dnmt1 is thought to be recruited to the replication fork after pre-RC activation via a PCNA interacting domain (Araujo et al, 2001; Iida et al, 2002). Furthermore, RNAi-mediated knock-down of DUE-B protein levels in HeLa cells had no effect on the level of Dnmt1 on chromatin (data not shown). Thus it remains to be determined precisely how DUE-B is recruited to replication origins and if the C-terminus plays a role in its function at the replication origin. Also to be investigated is whether the distribution of DUE-B along chromosomes is altered when it lacks the C-terminal DNA binding domain. It is quite possible that this domain allows DUE-B to recognize replication origins or DNA unwinding elements.

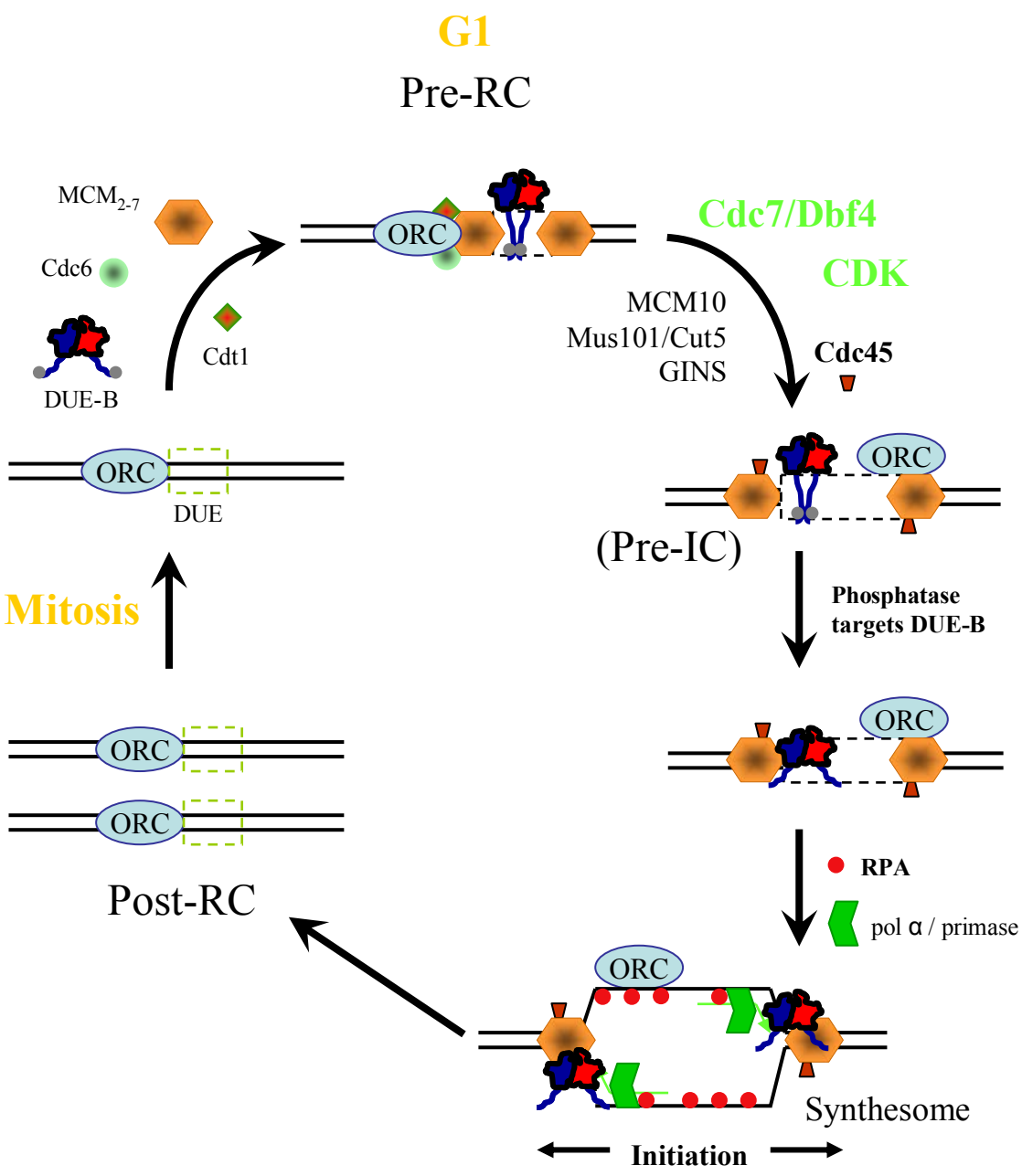
Incidentally, the yeast DTD1 protein was reported to interact in a yeast two-hybrid screen with the Rpb9 component of the RNA polymerase II complex (Ito et al, 2001), suggesting that the recruitment of the N-terminal DTD1-like domain of DUE-B to chromatin may occur independently of the C-terminal domain. Like DTD1, Rpb9 is non-essential in yeast, though Rpb9 is important for transcriptional fidelity and for stimulating Pol II release from the arrested state (Awrey et al, 1997; Nesser et al, 2006; Ziegler et al, 2003). Interestingly, in budding yeast RNA polymerase II (Pol II) could be crosslinked to the ARS1 replication

origin on plasmids, and the C-terminal domain (CTD) of Pol II alone can stimulate minichromosome maintenance (Gauthier et al, 2002). Moreover, in *Xenopus* oocytes and HeLa cells several members of the MCM complex were shown to co-purify with RNA polymerase II, specifically with the CTD (Yankulov et al, 1999). These results suggest a role for RNA polymerase II in the formation of pre-RCs at replication origins. The putative interaction of DUE-B homolog with a component of the RNA polymerase II complex might thus also be related to its replication origin binding activity.

Model for DUE-B in DNA replication

The data presented indicate an important function for DUE-B at chromosomal replication origins in both human cells and *Xenopus* egg extracts, suggesting a functional conservation of DUE-B in the replication of vertebrate genomes. Elimination of DUE-B from human cells or from *Xenopus* egg extracts significantly reduces or slows the rate of DNA synthesis. The discovery of DUE-B as a DNA unwinding element binding protein implies a function for DUE-B at the origin or at newly established replication forks. A model for DUE-B function in DNA replication is presented in Figure 64. In this model DUE-B is recruited to replication origins through an interaction with as yet unknown factors. Then, during the activation of pre-RCs and the recruitment of Cdc45 to the origin, DUE-B exerts its replication-promoting function via its C-terminal DNA binding domain. In this scenario, the DUE-B C-terminus may stabilize or promote a structural intermediate in the origin DNA that allows Cdc45 to bind the origin. Furthermore, the phosphorylation of DUE-B may be important in modulating this binding, such that DUE-B with an unphosphorylated C-terminus may inhibit this origin activation under certain conditions. Furthermore this dephosphorylation event may normally serve to prevent reinitiation at the replication origin during normal S phase. The activated origin then recruits the replication polymerization machinery to the origin, and DUE-B makes additional contacts with other protein components of the replication machinery as it moves with the replication machinery away from the origin as the chromosomal DNA is replicated.

Figure 64. Model for DUE-B function in DNA replication.



CONCLUSIONS

The results presented here demonstrate roles for two novel trans-acting factors at chromosomal origins of DNA replication in metazoans, both of which may promote replication origin activity. Increased histone acetylation promotes the establishment of replication complexes on the DNA, presumably through an increase in the accessibility of replication initiation proteins to the DNA. This then allows replication to initiate at new sites in the genome and at more potential sites within broadened zones of replication initiation. Furthermore, increased histone acetylation results in the firing of some replication origins earlier in S phase. A second factor, DUE-B, was identified based on its affinity for DNA with intrinsic helical instability, a common component of replicator elements. Elimination of DUE-B from either human cells or Xenopus egg extracts inhibits DNA replication and the entry of cells in to the S phase of the cell cycle. Although DUE-B contains an N-terminal domain identical to that of the D-aminoacyl-tRNA deacylase, the presence of a vertebrate-specific C-terminal domain that binds DNA suggests a novel function for this tRNA metabolizing protein in the replication of chromosomal DNA. Together these results suggest that numerous factors act to promote the assembly and activation of replication protein complexes on metazoan chromosomes.

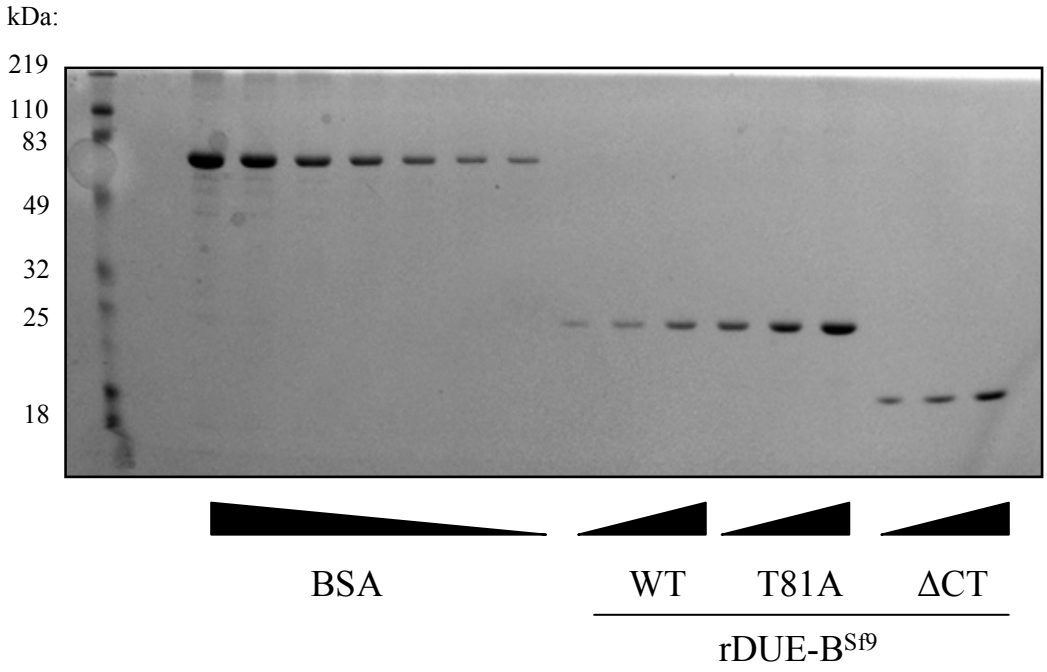
APPENDICES

Appendix A - Primer/oligonucleotide sequences

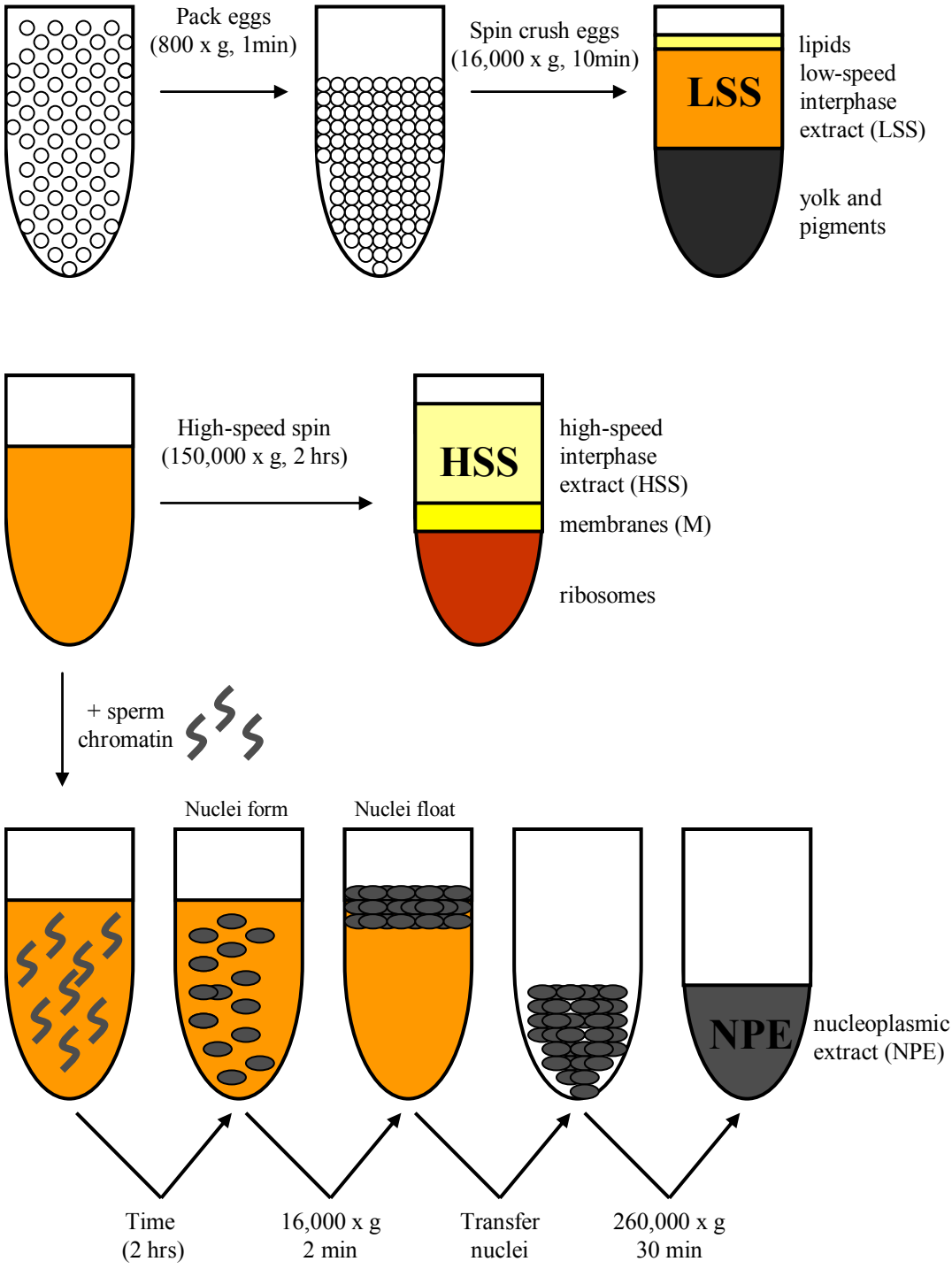
Oligo Name	Sequence of oligonucleotide
EMSA-DUE Lower	5'-TTGTTTCTCGAGCTCCCTGAAATGATCTA-3'
EMSA-DUE Upper	5'-GAAGGAATTCATGAGAAGAATGTTTTTTG-3'
DUE-BtoBlueBac-Upper	5'-ATCATATTGGATCCGATGAAGGCCGTGGTGCAG-3'
DUE-BtoBlueBac-Lower	5'-TAGTATACCGGTACCCGGCTCCCGTTCAGAGGA-3'
Thr81Ala-Upper	5'-CAGTTTGCACTCCAGTGTGTC-3'
Thr81Ala-Lower	5'-CTGGAGTGCAAACTGGCTGAC-3'
Cterm62aa-Lower	5'-AAGCATGACCGGTACCAGCTGGCGATTC-3'
siRNA_Mutant-Upper	5'-GGGAAACATTGGAGCAAATCCGTCATGGACAAACAGTACGAGA-3'
siRNA_Mutant-Lower	5'-GACGGATTTGCTCCAATGTTTCCCACTCTCATCCTCAAATA-3'
DUE-54nt-Upper	5'-AATAAAAAATCCCGAGGGAATATACATTATATATTAAATATAGATCATTTCAGG-3'
DUE-54nt-Lower	5'-CCTGAAATGATCTATATTTAATATATAATGTATATTCCCTCGGGATTTTTTATT-3'

Appendix B - Expression and purification of rDUE-B^{Sf9}

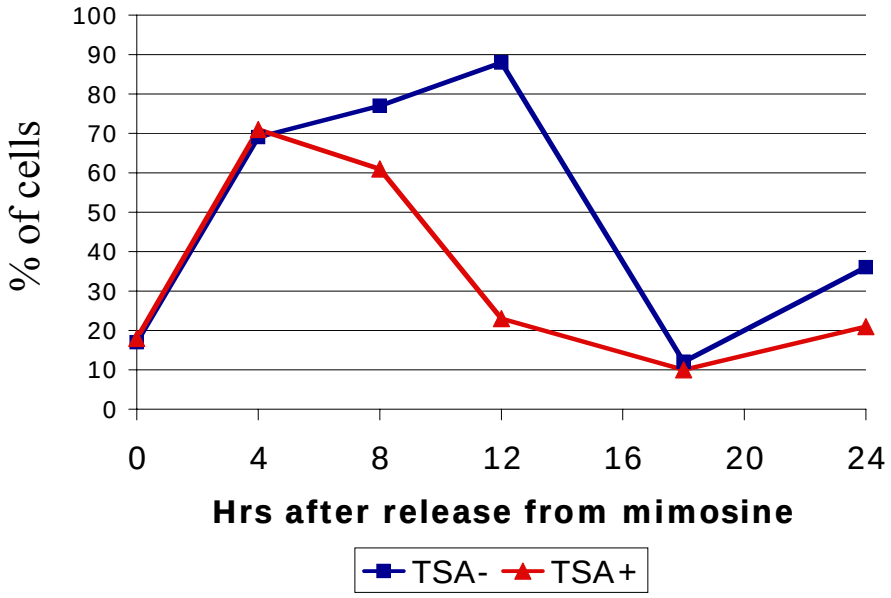
Baculoviruses encoding a wild-type, full-length form of human DUE-B, as well as a Thr81Ala and ΔCT mutant, were constructed for purification of recombinant protein. The figure displays a representative, coomassie stained gel of the purified proteins with BSA as a standard to estimate concentrations.



Appendix C - Preparation of *Xenopus* egg extracts



Appendix D – Quantification of cell cycle distribution from cells treated with TSA after release from a mimosine-mediated late G1-phase block.



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